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The Saga of a Male Fertility Protein (SP22)

Contents

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Summary

Toxicologic studies designed to identify chemical-induced alterations in the structure and function of the epididymis, particularly the acquisition of fertility by proximal cauda epididymal sperm, have lead to the discovery of a novel sperm protein (SP22) that is well correlated with the fertility of these "matured" sperm. SP22 is the first sperm-associated fertility protein identified following toxicologic investigation; a convincing demonstration that toxicology can be a tool to understanding fundamental physiology. SP22 is completely novel to reproductive biology, and has only recently been described (i.e. DJ-1/RS) in somatic tissues. The presence of a unique mRNA transcript in the testis, that appears around the time of round spermatid formation, together with the immunolocalization of SP22 on rete sperm, argue that SP22 is differentially regulated and expressed on maturing sperm in the testis following meiosis. With the generation of multiple antibodies to SP22 (i.e. peptide, recombinant) there is increasing evidence that the sperm-associated form of SP22 is expressed on the equatorial segment in rats, bulls, and humans. Since the addition of antibody to SP22 inhibits fertilization both in vitro and in vivo, it is tempting to speculate that SP22 is pivotal to initial sperm-egg binding. Unfortunately, as no function has been definitely established for the ubiquitously expressed somatic form, it is difficult to imagine what molecular role SP22 may play on the sperm membrane. While denatured, SP22 is one of several isoelectric variants at 28 kD, purification of native SP22 indicates a molecular weight maximum of approximately 450 kD. Thus, it appears that SP22 represents a single, charged molecule in a family of charged variants of identical molecular weight which collectively contribute to a multimeric complex. Presumably the expression of SP22 on a population of sperm as it relates to their fertility represents the relative percentage of SP22 (the pI 5.2, 28 kD variant) within this 450 kD complex. If so, the "function" of SP22 may well be defined by the function of the 450 kD membrane complex in the sperm membrane. The percentage of SP22 may contribute to the overall size of the multimeric complex, which in turn determines the degree to which the complex functions. By analogy it is well known that the activity of many enzymes (e.g. catalase, NADP-dependent malate dehydrogenase, formyltransferase) increases as the protein transitions to higher multimeric states (e.g. dimer to tetramer; Shimna, S. et al., 1998; Iglesias, A., 1990). While difficult and time-consuming, understanding the biological role of native SP22 on the sperm membrane is crucial to designing and using SP22, and/or target specific probes (i.e. nonprotein small molecules, recombinants, monoclonal antibodies), that might act as agonists or antagonists, and serve as potential therapeutic enhancers or contraceptives, respectively. As additional antibodies are generated to SP22 (i.e. monoclonals to SP22 recombinants, monoclonals to the native SP22 multimeric complex) we will begin to characterize the ontogeny of this protein in the testis, as well as its physiological regulation in the testis and epididymis. It is important to determine in which stage(s) of spermatogenesis

the germ cell specific SP22 transcript is expressed, which germ cells express it, the cellular localization within these cells, and if germ cell expression is hormonally regulated. Moreover, it is important to understand how SP22 expression is regulated (i.e. stabilized and/or modified) during epididymal transit. For example, it remains to be determined whether the change in pattern of immunolocalization observed for rete sperm and cauda sperm represents loss from one region and subsequent acquisition in another region, a transmembrane relocalization, or a masking and unmasking phenomenon. Only with this information will it be possible to determine SP22's potential as biomarker of toxicity in the adult testis and epididymis, as well as in the developing male reproductive tract. Finally, realizing the complete potential for SP22 as a biomarker of toxicity, as a fertility diagnostic, a therapeutic enhancer, or as a contraceptive, will be possible only once species differences and similarities, particularly with regard to ontogeny, regulation, and molecular function are known and understood.

Kew-words: SP22, male fertility protein, fertility.

Background - A novel sperm fertility protein - why and how?

Today there is heightened awareness to the possibility that environmental exposure to certain chemicals may be causal in the putative decline in semen quality in the human population over the past several decades (Giwercman & Skakkeback, 1992; Sharpe, 1993; Medical Research Council, 1995). At least some recognize the fact that a potential decline in semen quality may be linked to alterations in seminal fluid characteristics and sperm quality (Comhaire et al., 1995, 1996), as well as to a reduction in sperm number. Over the last 12 years my laboratory has been focused on the identification and characterization of a sperm membrane protein that might be applicable to a determination of whether there is a toxicologic link to diminishing human sperm quality; and if so, is the fertilizing potential of compromised sperm in jeopardy. We realized that male reproductive toxicology holds a key to unlocking new discoveries regarding the physiology of the "normal" reproductive process, including sperm proteins that are pivotal to the process of sperm maturation and the acquisition of fertilizing ability.

In recent years a considerable effort has been put forth by numerous laboratories attempting to identify and characterize sperm proteins that are functionally important to the process of fertilization. While several sperm proteins (O'Rand et al., 1984, 1996; Burks et al., 1995; Lea et al., 1996; Boue & Sullivan, 1996; Primakoff et al., 1997; Amann et al., 1999) as well as epididymal fluid proteins (Cuasnicu et al., 1984) and seminal plasma proteins (Killian et al., 1993, 1996; Peknicova et al., 1997) have been associated with fertility over the years, these proteins were initially identified based on their relative abundance in the sperm membrane or seminal plasma, or on the ability of antiserum raised against sperm membrane and seminal plasma protein preparations to recognize specific sperm antigens. Subsequent interest in many of these candidate fertility proteins has evolved following demonstrable

binding of these proteins to the investments of the eggs, or the ability of antibodies raised against these proteins to inhibit interaction with the egg and/or fertilizing ability either in vitro or in vivo. Some of these proteins (i. e. PH-20, LDH-C4, Fertilin, D/E) have been used fairly effectively as contraceptive antigens in animals (Primakoff et al., 1988; O'Hern et al., 1995, Chodavarapu et al., 1996; Ellerman et al., 1998). Recently, Fertilin B has been implicated with a direct role in sperm-egg interactions based on results obtained using mice that were lacking Fertilin B (Cho et al., 1998).

Unfortunately, candidate fertility proteins have rarely been identified during a physiological, i.e. in vivo, assessment of fertility. Truly important fertility proteins may represent only minor constituents of the sperm membrane protein profile; proteins that are likely to go undetected when antiserum is raised against a crude preparation of sperm membrane proteins, or against concentrates of luminal fluid or seminal plasma proteins. It is with this underlying premise that the quest for a novel sperm protein biomarker of fertility began in our laboratory.

It is now widely-accepted that the acquisition of fertilizing ability occurs within the epididymis. For many years, it has been thought that the process of epididymal sperm maturation involves a functional interaction between secretory activity of the epididymal epithelial principal cells, constituents of the epididymal fluid milieu, and the resident sperm (Bedford, 1975; Orgebin-Crist et al., 1975). To a large extent this notion arose from evidence that facets of epididymal sperm maturation proceed in an orderly fashion, with the acquisition for progressive motility preceding the acquisition of fertilizing ability. This, coupled with the fact that processes such as sperm egg binding are mediated by the action of androgens (i.e. testosterone and dihydrostestosterone) on the epididymis (Cuasnicu et al, 1984) , and that numerous epididymal secretory proteins do indeed associate with the sperm in the lumen of the epididymis (Kohane et al., 1980; Klinefelter & Hamilton, 1985), fostered the concept that requisite fertility proteins are incorporated by the plasma membrane of sperm during epididymal transit via the action of androgens. It therefore is quite disconcerting that to date few androgen-dependent epididymal secretory proteins that are actually incorporated into the sperm plasma membrane have been associated with the acquisition or maintenance fertilizing potential. One notable exception to this is the D/E protein complex described originally by Garberi et al. (1979) and Brooks and Higgins (1980). Rat epididymal protein D/E is synthesized under the influence of androgens by the epithelium in the proximal, i.e. caput/corpus, region of the epididymis and associates with the sperm membrane during epididymal transit (Kohane et al., 1980[a,b]). Moreover, this protein is thought to localize ultimately over the equatorial segment of the sperm head where it has been implicated in the acrosome reaction and sperm-egg fusion (Rochwerger & Cuasnicu, 1992; Cohen et al., 1996). While the data for this epididymis-derived sperm protein are suggestive of a pivotal role in fertilization, and recently immunizations with this protein complex has been shown to render rats infertile (Ellerman et al., 1998), it is questionable whether this protein has significant homologous structure and function across species.

We felt strongly that by carefully characterizing the toxicology of the epididymis, it would be possible to identify a sperm surface protein that could serve as a biomarker of fertility across species. To identify toxicant-induced alterations in sperm maturation, a testing strategy must be devised to optimize the likelihood of detecting an epididymis-specific alteration and simultaneously minimize the likelihood of detecting effects in the epididymis that could be considered secondary to a testicular insult. By taking advantage of the known time that sperm spend in transit within the epididymal duct en route from the testis to the vas deferens, toxicant exposures can be designed within the window of epididymal sperm maturation. In theory, epididymal sperm recovered within this maturation window would have resided solely within the epididymis, and not the testis, during the exposure period.

Upon exiting the testis and entering the proximal segments of the epididymis, i.e. the caput epididymidis, rat sperm typically require 4 days (Robb et al., 1978) to reach the proximal cauda epididymidis, the site where the first fertile sperm can be found (Figure 1A). If fluid dynamics within the lumen of the epididymis are constant, and sperm transit rate is unaltered, sperm undergoing processes of androgen-dependent maturation within the caput epididymidis at the onset of exposure, can be recovered in the proximal cauda epididymidis 4 days later. If toxicant exposure induces structural and/or functional changes within the epididymidis, and the process of maturation is in turn compromised, these sperm may indeed be immotile or infertile. With this logic we proceeded to test chemicals that, based on the early literature, were likely to alter sperm maturation within the epididymis within 4 days of exposure.

Ethane dimethanesulphonate (EDS), a chemical that was initially proposed for use as a cancer therapeutic agent was discovered to reduce fertility in rats two weeks following a single administration (Jackson, 1964). Subsequent studies demonstrated that a single administration of EDS resulted in a significant decline in circulating testosterone (T) levels within two days and that EDS was capable of selectively eliminating the Leydig cells within the testis (Jackson & Morris, 1977). Thus, despite several reports noting the formation of sperm granulomas in the epididymis, EDS-induced infertility was associated solely with deficits in spermatogenesis following Leydig cell toxicity and androgen withdrawal; the epididymis was never conceived as an alternate target.

Our laboratory was the first to explore the possibility that EDS might also exert effects on the structure and function of the epididymis, and that such effects might occur independently of Leydig cell toxicity and ensuing testicular compromise. By first administering EDS in a single dose on experimental day 1 and evaluating the structure and function of the epididymis four days later, we observed not only the formation of sperm granulomas in the caput epididymidis, but also significant histological alterations within the epithelium along the length of the epididymis, significant reductions in epididymal weight and cauda epididymal sperm number, significant changes in the motion parameters of proximal cauda epididymal sperm,

and diminutions in specific detergent-extracted sperm proteins (Klinefelter et al., 1990). To reduce the likelihood that these effects were attributed to the EDS-induced decline in serum T, or perturbations in the testicular fluid entering the epididymis, the protocol was expanded to include exogenous administration of T via subdermal T-filled Silastic implants, and surgical ligation of the efferent ducts, respectively (Figure 1B). While T administration alleviated the magnitude of the decrease in progressive motility and certain sperm proteins, neither T nor efferent duct ligation was able to prevent the formation of sperm granulomas and the marked histological alterations within the epididymal epithelium, the decrease in the velocity of sperm motion, or the diminution of specific sperm membrane proteins. Together, these findings strongly suggested that EDS was capable of exerting profound effects on the epididymis, effects which appeared direct rather than secondary to the testicular insult. Using a novel method for the coculture of immature sperm and epididymal epithelial cells we were able to demonstrate that direct exposure to EDS does indeed alter epididymal epithelial cell function and facets of sperm maturation such as the acquisition of secreted protein by sperm, and the acquisition of the capacity for progressive motility (Klinefelter et al., 1992).

With the demonstration that EDS was a direct acting epididymal toxicant we extended our experimental strategy to other putative epididymal toxicants, i.e. chloroethylmethanesulphonate (CEMS), epiclorohydrin (EPI), and hydroxyflutamide (HFLUT). Both CEMS and EPI are structural analogues of ethylene gylcol. Like EDS, CEMS also was initially proposed for use in cancer therapy, and it likewise caused transient infertility in male rats within a couple weeks of administration (Jackson et al., 1961). Earlier work provided data suggesting that either (α-chlorohydrin or EPI compromise fertility (Tsunoda & Chang, 1976; Toth et al., 1989), but attempts to delineate testicular from epididymal toxicity had not been made. To evaluate the antiandrogenic consequences of exposure to the synthetic antiandrogen HFLUT on the epididymis, we used a castrate, T-implanted animal model (Figure IC) . The exogenously-administered T is sufficient to maintain the maturation of sperm residing in the caput epididymidis at the time of surgery such that 4 days later, these sperm now in the proximal cauda epididymidis, have complete fertilizing ability (Dyson & Orgebin-Crist, 1973; Klinefelter et al., 1994). However, removal of the testicular supply of androgen, reduces the level of endogenous ligand within the epididymis, thereby facilitating the action of the antiandrogen (Klinefelter et al., 1994).

Prior to evaluating the effects of these putative epididymal toxicants on fertility and associated male reproductive endpoints, we needed to choose a method to assess fertility. is well known that the process of spermatogenesis is highly efficient in the rat and other laboratory animals relative to humans (Amann, 1986). Indeed, it requires a robust toxic insult to observe a deficit in fertility in male rats that are allowed to mate naturally, and typically these insults produce quantitative as well as qualitative alterations in the ejaculate . Much of the relative difference in efficiency in rats

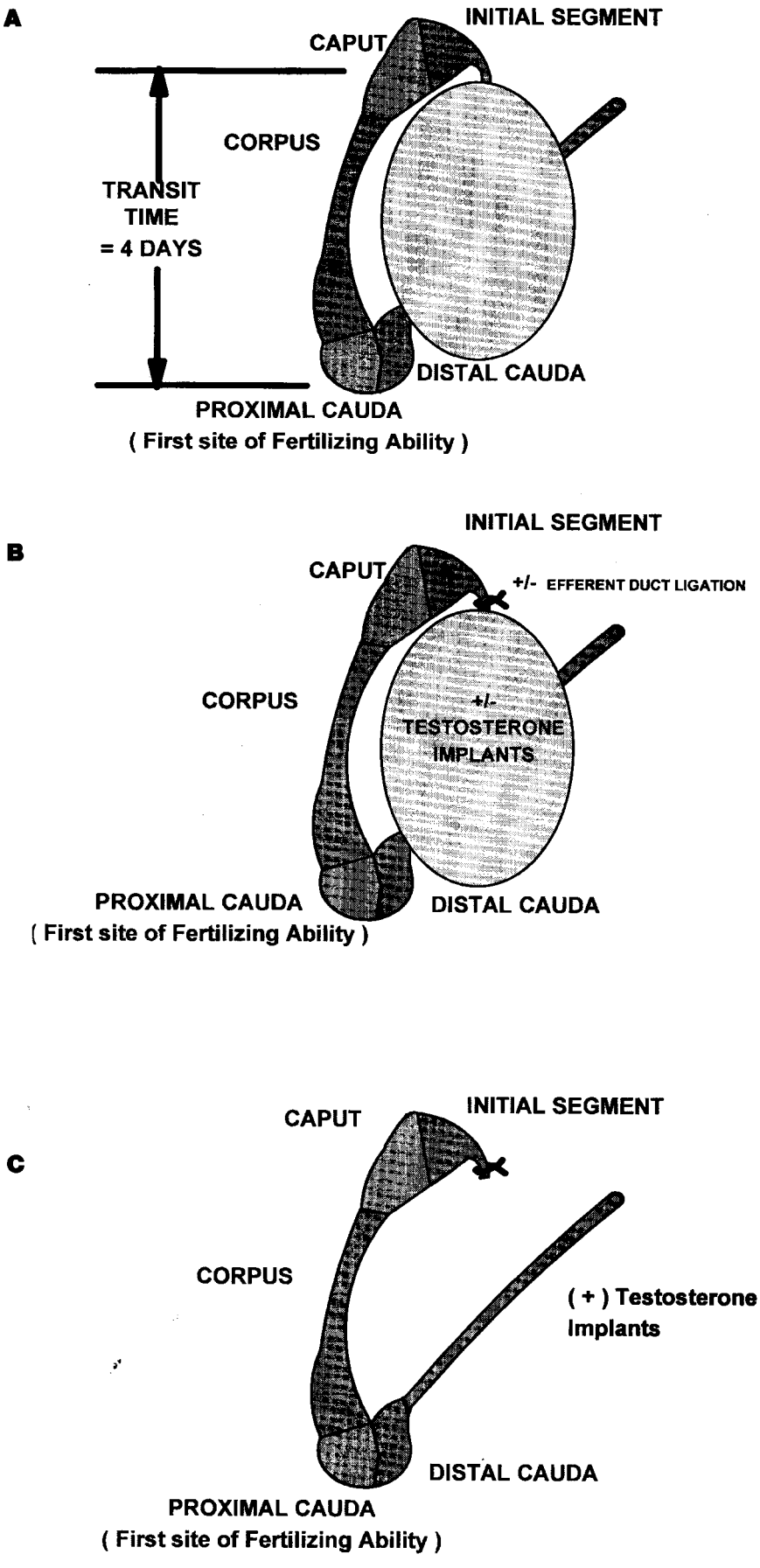


Figure 1. Schematic diagram to depict the experimental strategies used to identify epididymal toxicants. A) Sperm that are resident in the caput epididymidis at the onset of exposure can be recovered from the proximal cauda epididymidis 4 days later. Sperm in the proximal cauda are more homogeneous with respect to their resident age than those in the distal cauda, and therefore afford increased sensitivity. B) Toxicant-induced reductions in circulating testosterone (T) can be prevented by subdermal administration of a 2.5 cm T-filled Silastic implant in the subscapular region. This will ensure that the epididymis receives adequate T from the circulation to support sperm maturation over 4 days. In addition, perturbations in testis-derived, luminal fluid constituents can controlled for by surgical ligation of the efferent ducts. C) To demonstrate anti-androgenic alterations in epididymal function within 4 days, testes are removed and a maintenance level of T is achieved via T implantation.

compared to men is attributable to a higher rate of germ cell atresia in the human testis, and coupled with this, is the fact that there is an unusually high incidence of morphologically abnormal sperm in a "normal" human ejaculate (Wyrobek et al., 1982). Thus, to identify subtle toxicant-related alterations in fertility in the rat that might provide credence for similar alterations in fertility in a "susceptible" (i.e. human) population, we elected to resurrect the technique of artificial (in utero) insemination (Dyson & Orgebin-Crist, 1973; Tsunoda & Chang, 1976).

First, we determined that 5 x 10^6 proximal cauda epididymal sperm would be the optimum, critical number of sperm needed for insemination of each uterine horn. This sperm dosage resulted in approximately a 75 % fertilization rate; a rate that was on the linear (i.e. responsive) region of the curve (Figure 2). Thus, 10 x 10^6 sperm were used for each insemination, regardless of whether the sperm were derived from control, untreated or toxicant-treated male rats. Next, we selected two doses of each of the putative epididymal toxicants; one that we knew from preliminary studies would affect changes in the epididymis, and another which we felt may or may not elicit alterations. Four days after the onset of dosing, in utero inseminations were performed and assessments of multiple endpoints were initiated (Figure 3). It seemed reasonable to suspect that some endpoints (e.g. specific sperm motion parameters, epididymal sperm numbers) might be preferentially compromised by one toxicant, while others (e.g. tissue T concentration, specific sperm proteins) might be altered by another toxicant. Ideally, if each toxicant resulted in a reduction in fertility, the correlations and relationships between specific endpoints (individually and in combination) and fertility could be established.

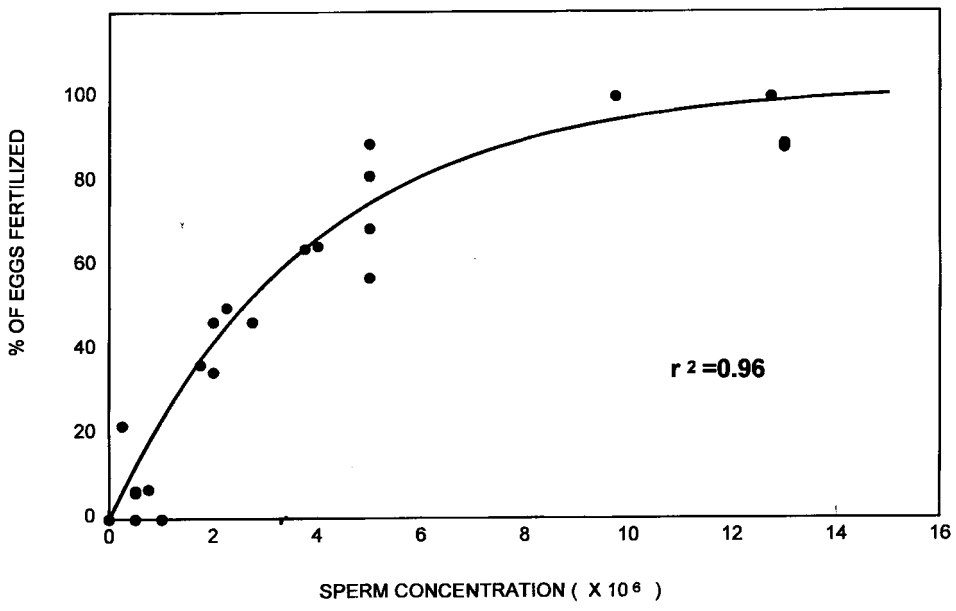
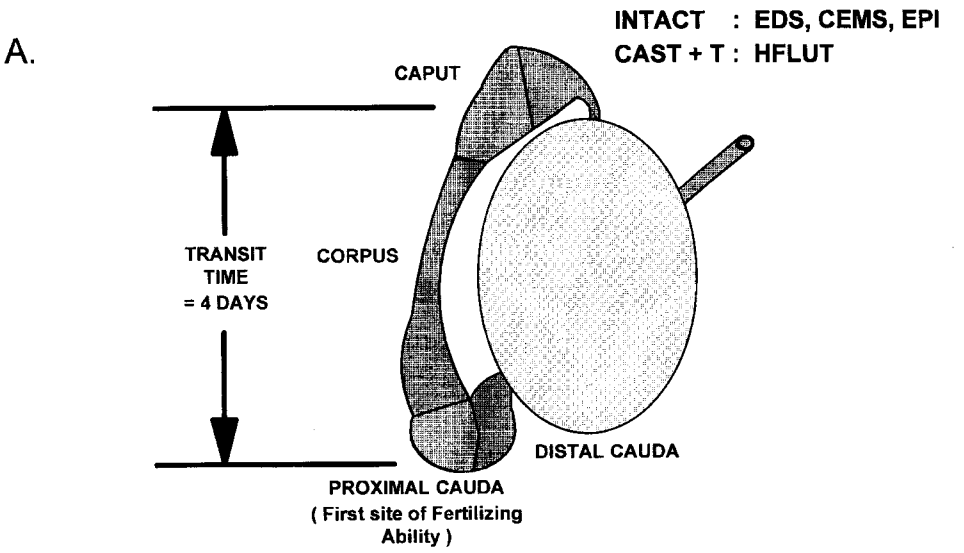


Figure 2. Increasing numbers of sperm from intact controls were inseminated in utero, eggs were later flushed from the oviducts, and fertilization rate was determined by the percentage of eggs containing a sperm tail and 2 pronuclei. Data were fit to a non-linear equation [Fertility = Max Fert. x (1 - $e^{-k \times s}$), where Max = 104.3 and k = 0.261]. Based on the fit of the data, insemination of 5 x 10^6 sperm per uterine horn would result in approximately 75 % fertilization; a region on the response curve where the slope is potentially sensitive to perturbation.



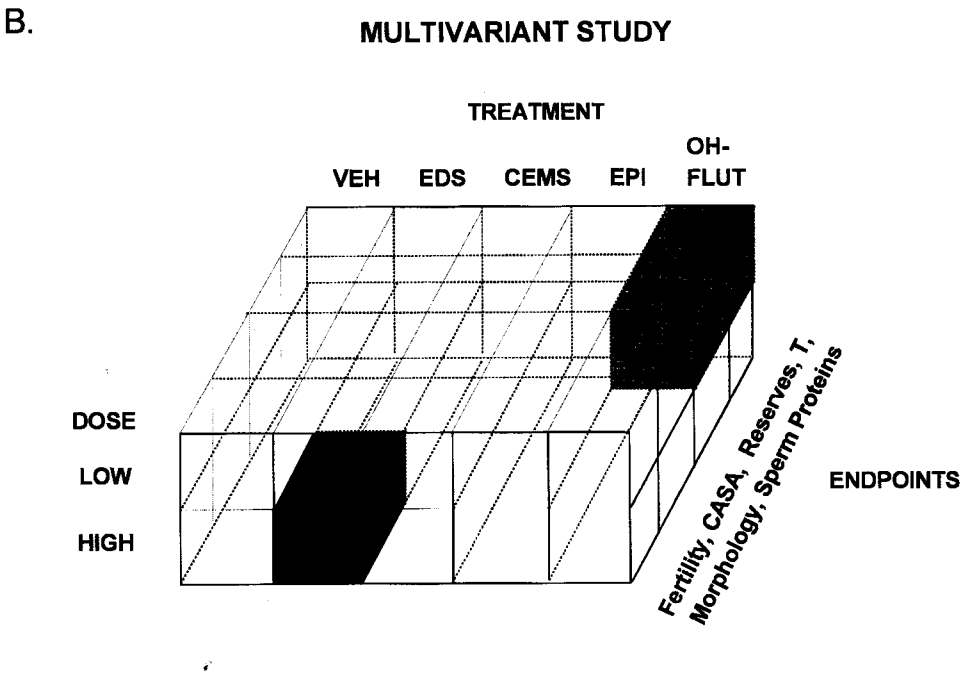


Figure 3. A) The experimental design was similar to that already discussed, but consisted of two animal models; an intact group, and a castrate, T-implanted group. Intact animals were used for EDS, CEMS, and EPI exposures, while castrate, T-implanted animals were used for HFLUT exposure. EDS was administered as a single ip injection on experimental day 1; CEMS and EPI were administered via ip injection daily for 4 days; and, HFLUT was administered via ip injection daily for 5 days. On experimental day 5 (4 days after dosing initiated), proximal cauda sperm were assessed. B) Each of the epididymal toxicants was administered at either a "low" or "high" dosage and multiple endpoints including fertility, sperm motion, sperm morphology, sperm reserves, and sperm membrane proteins were evaluated. It was anticipated that while fertility might be compromised by each toxicant, that the most sensitive endpoint would prove to be chemical specific.

Discovery of SP22

Fertility, assessed as the number of gestation day 9 implants relative to the total number of corpora lutea, was significantly decreased following 4 days of exposure to each of the epididymal toxicants (Klinefelter et al., 1997). Moreover, while the higher dose of each chemical was effective, the lower dose of HFLUT and EPI compromised fertility as well. Of the multiple endpoints that were evaluated, only 2 correlated significantly with fertility; T levels in the caput/corpus epididymidis ($p < 0.02$; $R = 0.32$) and a sperm protein referred to as SP22 ($p < 0.0001$; $R = 0.76$). Clearly, the SP22 quantified in silver-stained gels following 2 dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D SDS PAGE) of detergent sperm extracts (Figure 4) provided the strongest association with the fertility of inseminated sperm. Interestingly, of the 120 protein spots that were resolved in the gels of control sperm extracts, only SP22 was altered by toxicant exposure in both a treatment and dose-related fashion. In fact, the relative changes in mean SP22 levels across treatment groups mirrored the relative alterations in fertility. Thus, while the correlation of this single protein spot with fertility appeared striking, it was quite consistent with the observed treatment related diminutions in both fertility and the SP22 quantified in 2D gels. While it could be argued that fluctuations in sperm SP22 levels reflect changes in sperm viability, we found that in general, sperm motion parameters were not significantly compromised by the doses of the toxicants in this study. In fact, we intentionally selected doses that were deemed unlikely to alter sperm motility to optimize the likelihood of detecting novel, more sensitive indicators of fertility.

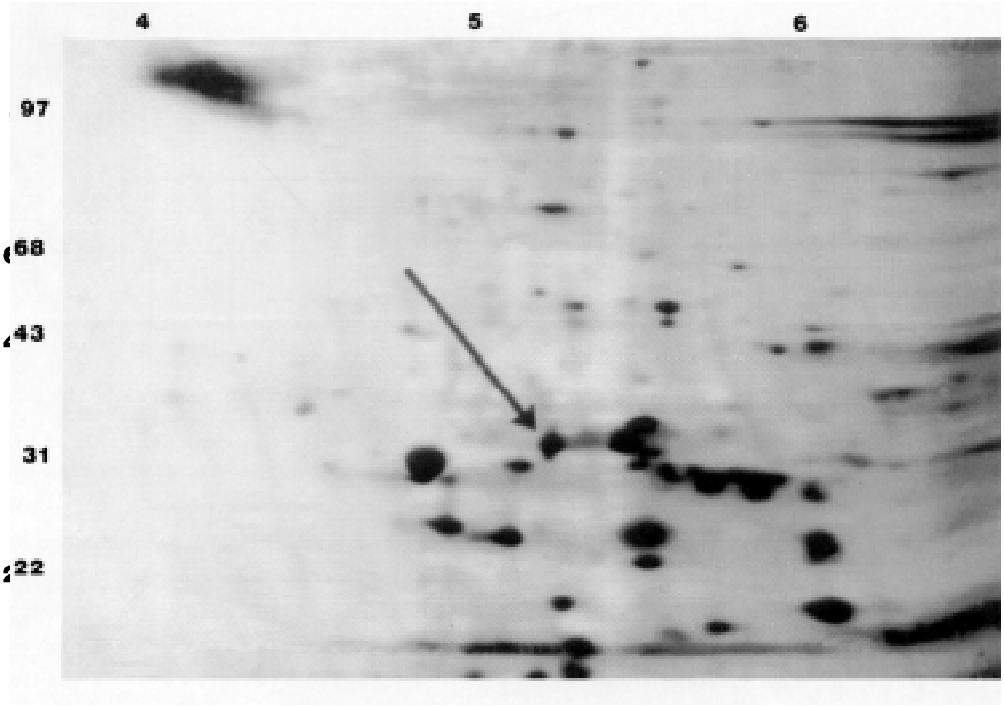


Figure 4. Profile of silver-stained proteins in a detergent extract of proximal cauda epididymal sperm resolved by two-dimensional SDS-PAGE. The isoelectric range is indicated across the top and the apparent molecular weights are indicted on the left (kD). The SP22 protein (28 kD, pI= 5.2) is indicated (arrow).

Upon examination of a scatter plot depicting SP22 levels and fertility for the sperm from each animal in the study regardless of treatment (Figure 5) it appeared that a nonlinear relationship existed. The data suggested a threshold amount of SP22 might be requisite for maximum fertility and therefore a sigmoid curve model was applied. In this model there are two rate constants; one which expresses the increase in fertility as SP22 levels are increased, and another which expresses the declining increase once a threshold level of SP22 is attained. Indeed, the correlation (r^2) when data were fit to this equation was 0.83. Moreover, it was apparent that the predicted threshold matched the actual threshold region of the curve. When the background-corrected, integrated optical density of SP22 was less than 10,000 units, fertility was less than 50 %; when SP22 levels ranged between 10,000 and 1 1,000 units, the resultant fertility could range from O - 70 %; and when SP22 levels exceed 1 1,000 units, fertility was greater than 50 %.

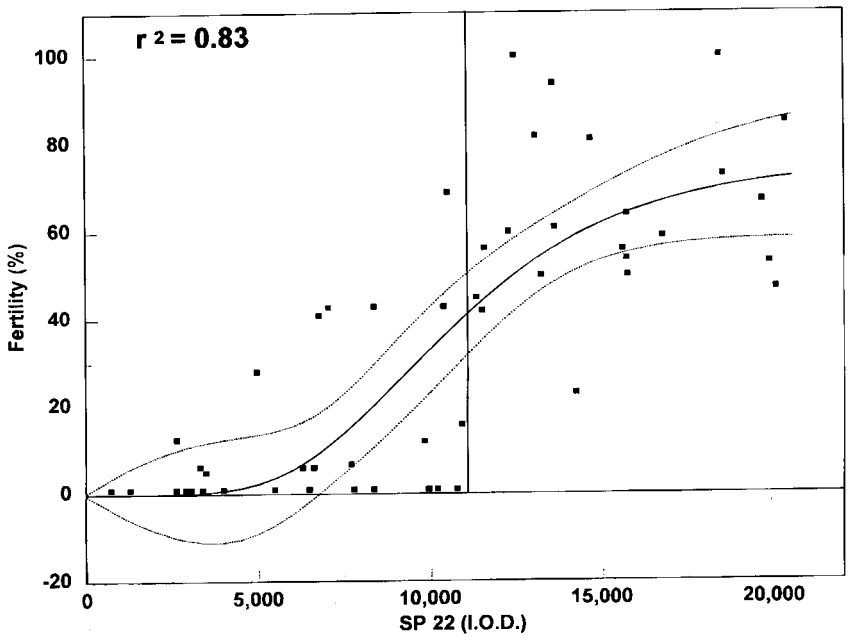


Figure 5. A scatter plot showing the relationship between fertility and the integrated density of SP22 for 50 animals $F_{SP22} = F_0 \exp^{A/B(1-\exp^{-B*SP22})}$ in which F_{SP22} is the fertility at protein concentration **SP22**, F_0 is the fertility a 0 protein concentration. **A** and **B** are constants (A is the initial increase in fertility and B is the rate of exponential decay of the increase in fertility). The correlation was highly significant ($P < 0.0001$) with an $r^2 = 0.83$. The dotted lines represent the 95 % confidence limit around the fitted line. Notice that when SP22 levels reach a "threshold" value of 11,000 fertility ranges from 0 to 70 %. When SP22 levels are less than this threshold, fertility is typically less than 50 %, and when SP22 exceeds this threshold, fertility is consistently greater than 50 %.

The concept that a requisite, threshold amount of a factor such as SP22 might be associated with the fertility of sperm was not novel. It had previously been suggested (Amann & Hammerstedt, 1993; Amann, 1993) that optimal fertility is likely to involve the orchestration of the acquisition and/or modification of multiple factors (i.e. proteins, lipids), and that each such factor might have its own "set point" or threshold. In the case of SP22 it seems that while 10,000 - 11,000 units of SP22 was " required " for optimal fertility, there were some animals in which this level did not result in optimal fertility, suggesting that threshold amounts of other requisite factors had not yet been attained or maintained. However, while this explanation seems quite plausible, the possibility exists that the threshold relationship observed between SP22 and fertility is totally independent of other putative fertility factors. It is equally likely that the relationship is attributable to the structure and function of SP22 itself. Based on our work with affinity-purified, anti-SP22 peptide antibody (discussed below), we know that multiple charged variants of the 28 kD SP22 molecule exist. Moreover, more recent work in our laboratory demonstrates that native SP22 has a maximum apparent molecule weight of 450 kD, with peaks at 260 and 145 kD also resolved (Figure 6). Together, these data suggest that SP22 represents a single, charged variant in a multimeric protein complex. Thus, it is possible that optimal fertility of sperm is conferred by a transition from a smaller to a larger native multimeric species, which in turn results in a concomitant increase in molecular function and fertility.

Partial Characterization of SP22

SP22 was identified as a spot on silver-stained gels, so it was no surprise when it was not visualized with Coomassie Blue, the stain routinely used for peptide identification by Edman degradation and sequencing. Reversed phase HPLC was used to obtain sufficient enrichment to visualize SP22 in gels with Coomassie Blue. For this, n-octyl-B-glucopyranoside extracts of cauda epididymal sperm were concentrated and desalted. Following centrifugation, supernatants (2 mg) were lyopholized, and proteins solubilized in 5 % acetonitrile were injected onto a reverse phase C4 high performance liquid chromatography (HPLC) column. Fractions highly-enriched in SP22 were obtained using a linear gradient of 20-80 % acetonitrile in water with 0. 1 % trifluoroacetic acid. The SP22-containing fractions (42-44 % acetonitrile) were pooled, and aliquots equivalent to 60 ug protein were subjected to 2D SDS-PAGE. Following staining with Coomassie blue, the SP22 spots were punched out of the gels and frozen for subsequent peptide sequencing; punches were also made in unstained areas of the gel to provide background reference. Two separate sets of samples, each consisting of 40 SP22 punches and 40 blank punches, were used for sequence determination. Isolated SP22 punches were subjected to tryptic digestion and peptide separation by HPLC. Homogeneous peptide peaks were selected for sequence determination by Edman degradation and matches for the resulting sequences were determined using the BLAST protein database.

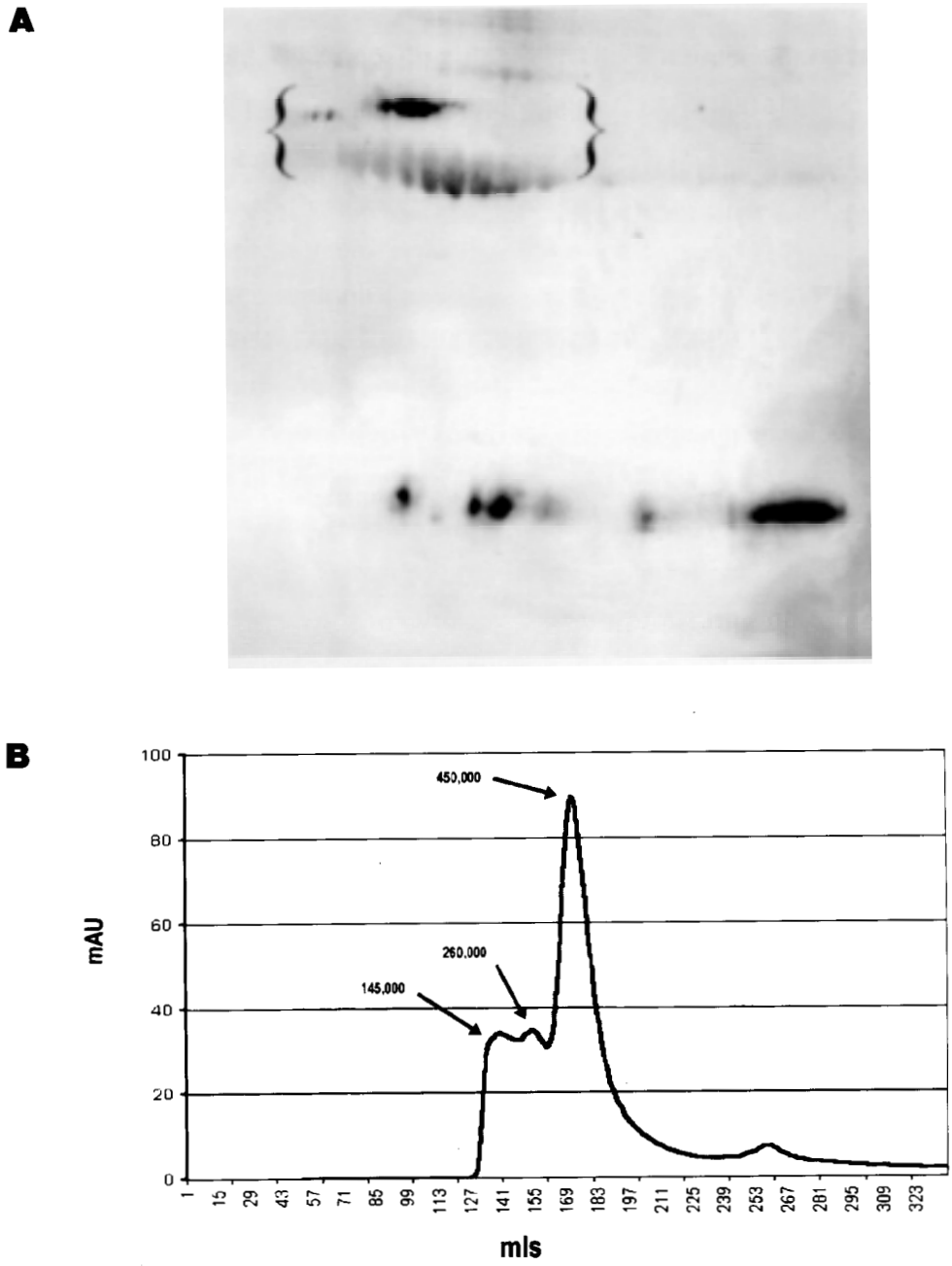


Figure 6. **A)** Profile of native SP22 following denaturation by 2D SDS PAGE. Multiple charged variants at 28 kD are observed, as well as heterogeneously-charged clusters of protein at much higher molecular weights (). All of these proteins are recognized on immunoblots using affinity-purified anti-SP22 peptide antibody (not shown). **B)** A chromatogram of native SP22 resolved on a Superdex-200HR (high resolution size exclusion) column. Three peaks with apparent molecular weights of 450 kD, 260 kD, and 145 kD are resolved.

Five tryptic peptide sequences were obtained (Welch et al., 1998) from rat SP22 (Figure 7), each matching only one known protein, designated DJ-1 (Nagakubo et al., 1997). Of these peptides, four matched the human DJ-1 amino acid sequence perfectly and another matched 5 of 7 of the comparable DJ-1 amino acids; the serine and glycine replaced threonine and histidine. As shown later, the glycine residue was errant, but the serine to threonine substitution is species related.

A 15 mer N-terminal peptide corresponding to the Edman degradation peptides #1 and #2, and an 8 mer C-terminal peptide corresponding to Edman degradation peptide #5, were used together as antigen to generate polyclonal antiserum in two Border Leicester Merino Sheep. Once obtained, immune serum was affinity-purified on a thiopropyl-Sepharose 6B column to which theses peptides were cross-linked. Affinity purified, anti-SP22 peptide immunoglobin (2 ug/ul) was first used to

A) Peptide # 1: **VTVAGLAGK**; Peptide #2: **DPVQCSR**; Peptide #3: **EILK**; Peptide #4: **TSGPLAK**; and Peptide #5: **DGLILTSR**.

B)

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1     MASKRALVILAKGAEEMETVIPVDVMRRAGIKVTVAGLAGKDPVQCSRDV 50
                                      Peptide  Peptide
                                         1        2

51    VICPDASLEDAKKEGPYDVVVLPGGNLGAQNLSESAAVKEILKEQENRKG 100
                                               Peptide
                                                  3
                                  * ●
101   LIAAICAGPTALLAHEIGCGSKVTTHPLAKDKMMNGGHYTYSENRVEKDG 150
                               Peptide
                                  4

151   LILTSRGPGTSFEFALAIVEALNGKEVAAQVKAPLVLKD 189
      Peptide
        5
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Figure 7. A) SP22 peptides identified by Edman degradation. B) full length amino acid sequence of human DJ1. (*) indicates that one Threonine (T) in the DJ1 sequence is substituted by a Serine (S) in the SP22 peptide # 4 sequence. (●) indicates that the Glycine (G) in the SP22 peptide # 4 sequence was later proven by cDNA sequencing to be an error and is shown correctly as Histidine (H) in the DJ1 sequence.

localize SP22 on Western blots of detergent extracts of sperm and tissues, and later on the plasma membrane of fixed and fresh sperm (Welch et al., 1998). Immunostaining on blots of detergent extracts of cauda epididymal rat sperm reveals that the SP22 peptide antibody recognizes the SP22 peptide and several charged variants of similar size (Figure 8). The fact that all immunostaining is ablated by addition of competing antigenic peptide (15 mer + 8 mer) to the antibody suggests that SP22 is one molecule in a heterogeneously-charged family of structurally-similar proteins. The notion that SP22 is present on the plasma membrane of cauda sperm was supported by the identification of SP22 in cauda sperm membrane preparations (figure 9). Moreover, an evaluation of protein in detergent extracts of sperm collected from the rete testis 18 hours following efferent duct ligation, provided the initial data indicating that SP22 originates on sperm in the testis.

Secondary structure predictions of the SP22 amino acid sequence identified three regions with putative alpha-helical conformation although none had sufficient hydrophobicity to be recognized as a typical transmembrane domain (Figure 10). It is possible that the association of SP22 with the sperm membrane is mediated by postranslational modification of the protein, possibly by the covalent addition of a lipid moiety. Seven myristylation sites (M) are present in the sequence which could allow SP22 to be tethered to the membrane by the addition of myristic acid in a manner similar to the src oncogene (Kaplan et al., 1990). Post translational modifications may also be responsible

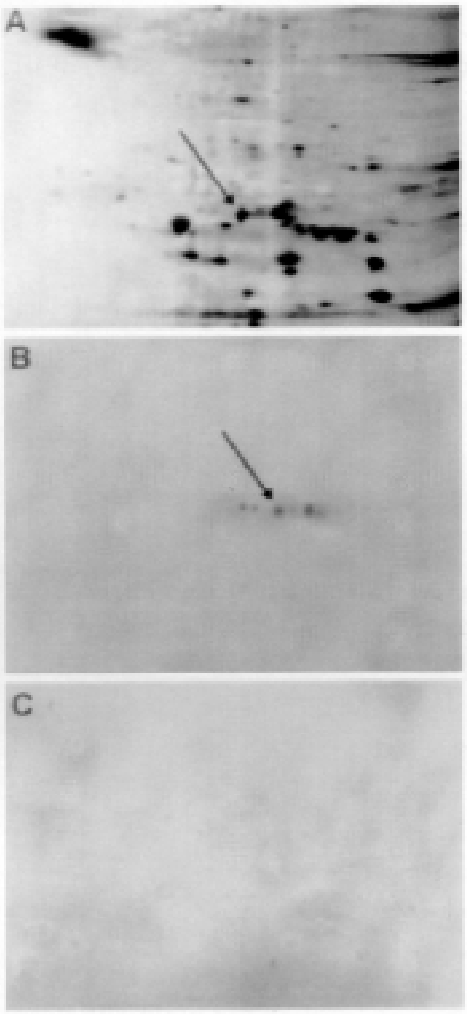

Figure 8. A) Silver-stained two-dimensional SDS PAGE (14% resolving gel) of proteins in a detergent extract of proximal cauda epididymal sperm. The arrow indicates SP22. B) The corresponding immunoblot. Note that the affinity-purified anti-SP22 peptide antibody recognizes SP22 as well as charged variants of similar molecular weight. C) There is no immunorecognition when SP22 peptide is added to the antibody.

for the pattern exhibited by SP22 on 2D SDS-PAGE. Nine phosphorylation sites targeted by casein kinase II (C) or by protein kinase C (P) are present in the sequence and could explain the observed charge heterogeneity of SP22. Two asparagine-linked glycosylation sites (N) are also present, but it is unclear if glycosylation actually occurs in this protein. While the presence of these modifications needs to be confirmed experimentally, they could provide a means to modulate both the localization and function of the SP22 molecule.

FITC localization of SP22 on sperm using the affinity-purified antibody indicated that SP22 resides over the anterior-ventral surface of the rat sperm head (Figure 11). The intensity and pattern of immunolocalization is unchanged whether sperm are fixed, fixed and detergent-permeabilized, or fresh. Moreover, the inununostaining appears specific to the 15 mer peptide as co-incubation of 8 mer peptide and antibody fails to reduce the FITC signal, but co-incubation of 15 mer peptide does reduce the signal (data not shown). Collectively, these data suggest that SP22 is present on the sperm membrane, with at least the N-terminal region of SP22

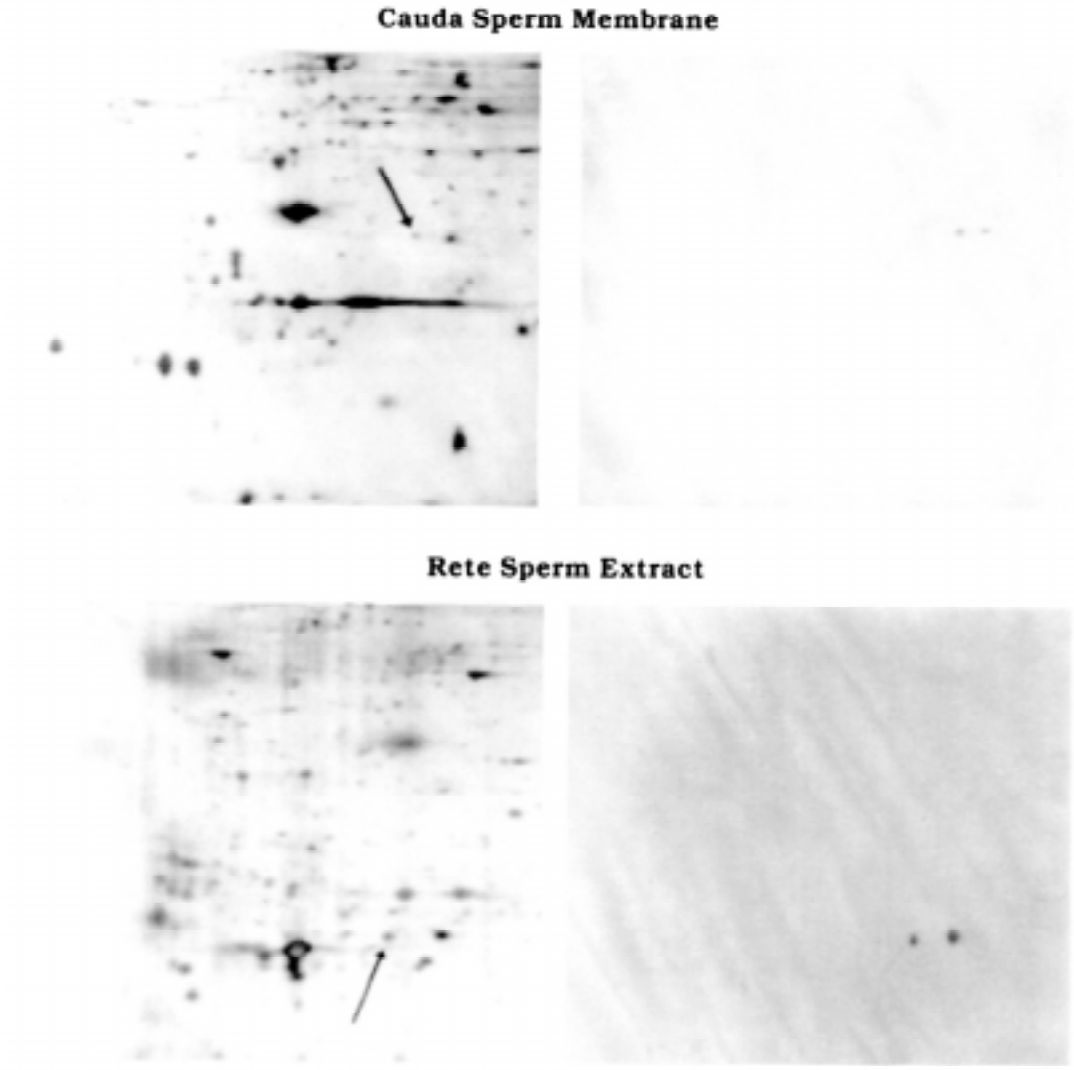

Figure 9. Silver stained 2D SDS gel profiles of proteins in a membrane preparation of sperm from the proximal cauda epididymidis, as well as in a detergent extract of sperm collected from the rete testis 18 hours after ligation of the efferent ducts. The SP22 protein is indicated with an arrow. The corresponding immunoblot for each preparation is shown on the right. Note that once again charged variants of SP22 exist.

containing the 15 mer sequence presented in an extracellular domain, where it is freely exposed and uncompromised by fixation.

Confirmation of the testicular origin of SP22 was also provided by immunocytochemical staining. As shown in Figure 12 an intense signal is found at the base of the head on sperm recovered from the rete testis 18 hours after ligation of the efferent ducts. In the caput/corpus epididymidis there is a translocation of the SP22 signal, with some staining at the base of head and some staining over the anterior-ventral surface of the head. By the time sperm reach the cauda, staining is consistently observed over the anterior-ventral surface of the head.

The similarity between the Edman degradation-derived sequences and the human DJ-1 protein suggested that SP22 and DJ-1 might belong to a similar class of proteins. A search of the GenBank database revealed a mouse homologue of DJ-1 containing the complete coding region which was used as a probe for cDNA library screening. Since SP22 was present on spermatozoa in the rete testis, implying

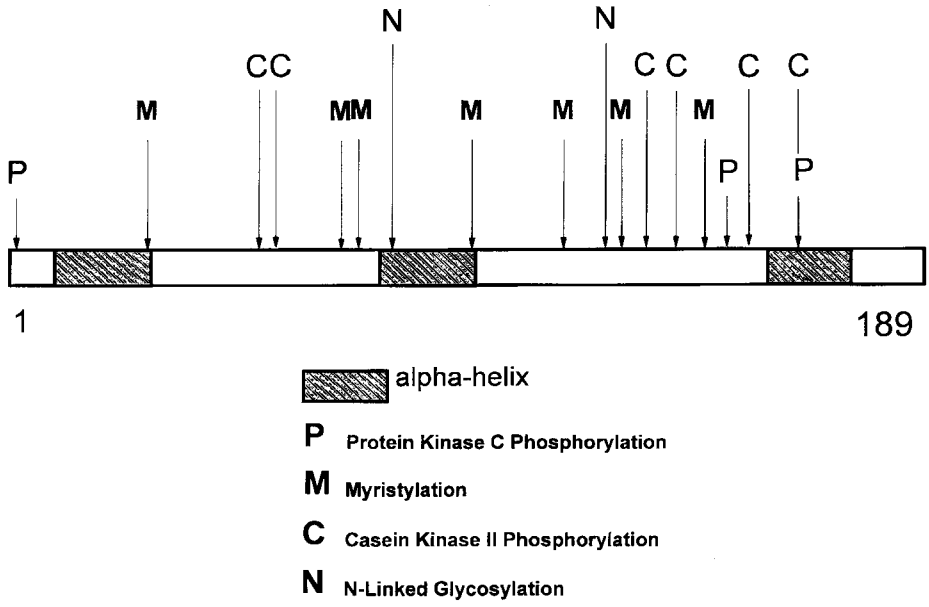

Figure 10. Predicted secondary structure and posttranslational modification sites of SP22. The 189 amino acid sequence of SP22 was screened for motifs indicative of possible secondary structure and postranslational modification sites. While three regions with putative alpha-helical conformation were identified (cross hatched), none had sufficient hydrophobicity to be recognized as a typical transmembrane domain. However, the seven myristylation sites (M) in the sequence could allow SP22 to be tethered to the membrane by the addition of myristic acid. The nine phosphorylation sites targeted by casein kinase II (C) or by protein kinase C (P) could explain the charge heterogeneity of SP22 observed by two dimensional electrophoresis. Two asparagine-linked glycosylation sites (N) were also present.

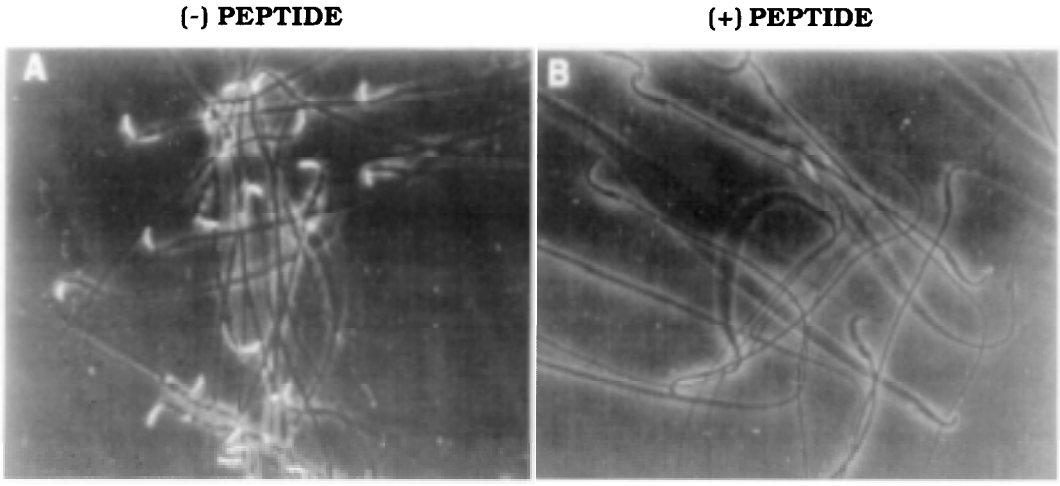

Figure 11. A) Immunolocalization of FITC on fresh, washed cauda epididymal sperm using affinity-purified anti-SP22 peptide antibody. Note that the SP22 localization is specific to the anterior-ventral aspect of the rat sperm head. **B)** Coincubation of sperm with the antibody and SP22 peptides to which the antibody was generated ablates all localization.

expression of SP22 in the gonad, a rat testis library was screened for the presence of SP22 sequences. The resulting cDNA clones fell into two groups (i.e. SP22A and SP22B; Figure 13) whose sequences differed only in their 5' untranslated region (Welch et al., 1998; Welch et al., 1999). The predicted protein sequence was identical for both transcripts. The sequences of the Edman-derived peptides #1, 2, 3, and 5 of tryptic digests of SP22 were shown to match tryptic peptides of the encoded protein perfectly, confirming the isolation of SP22 CDNA clones. For sequence containing peptide #4, six of seven amino acids matched the predicted rat SP22 sequence, containing the predicted serine in place of threonine; glycine was an Edman degradation sequencing error.

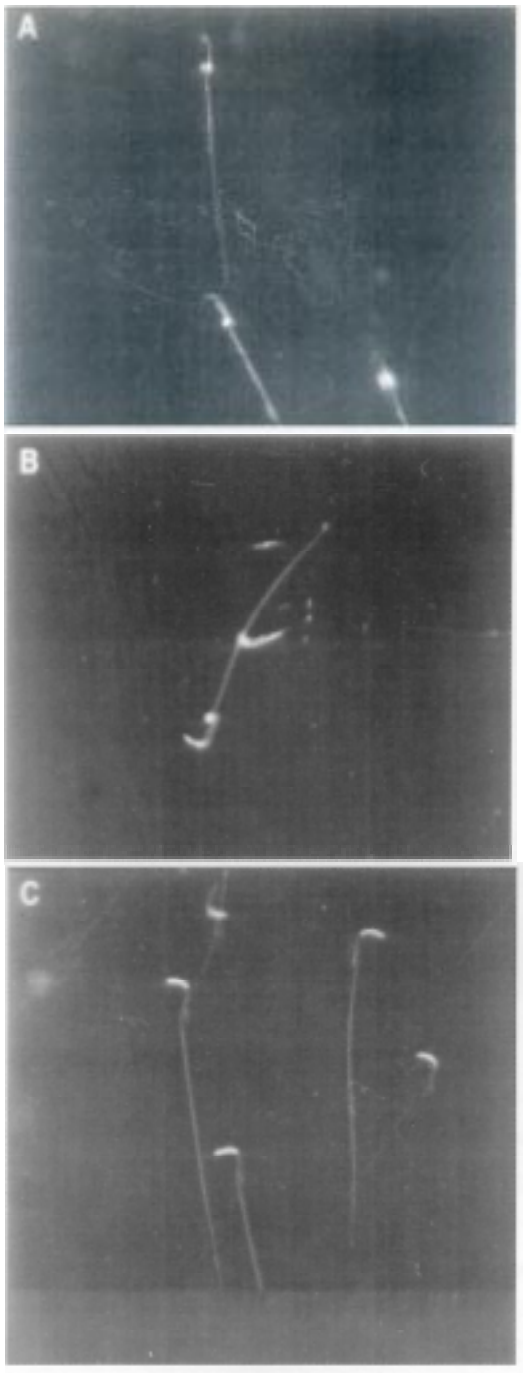

Figure 12. Micrographs depicting immunolocalization of SP22 on sperm from A) the rete testis, B) the corpus epididymidis, and C) the cauda epididymidis. The fluoresence is restricted to the base of the head when sperm exit the testis, but by the time they reach the corpus there is equal distribution between staining at the base of the head and staining over the anterior-ventral surface of the head.

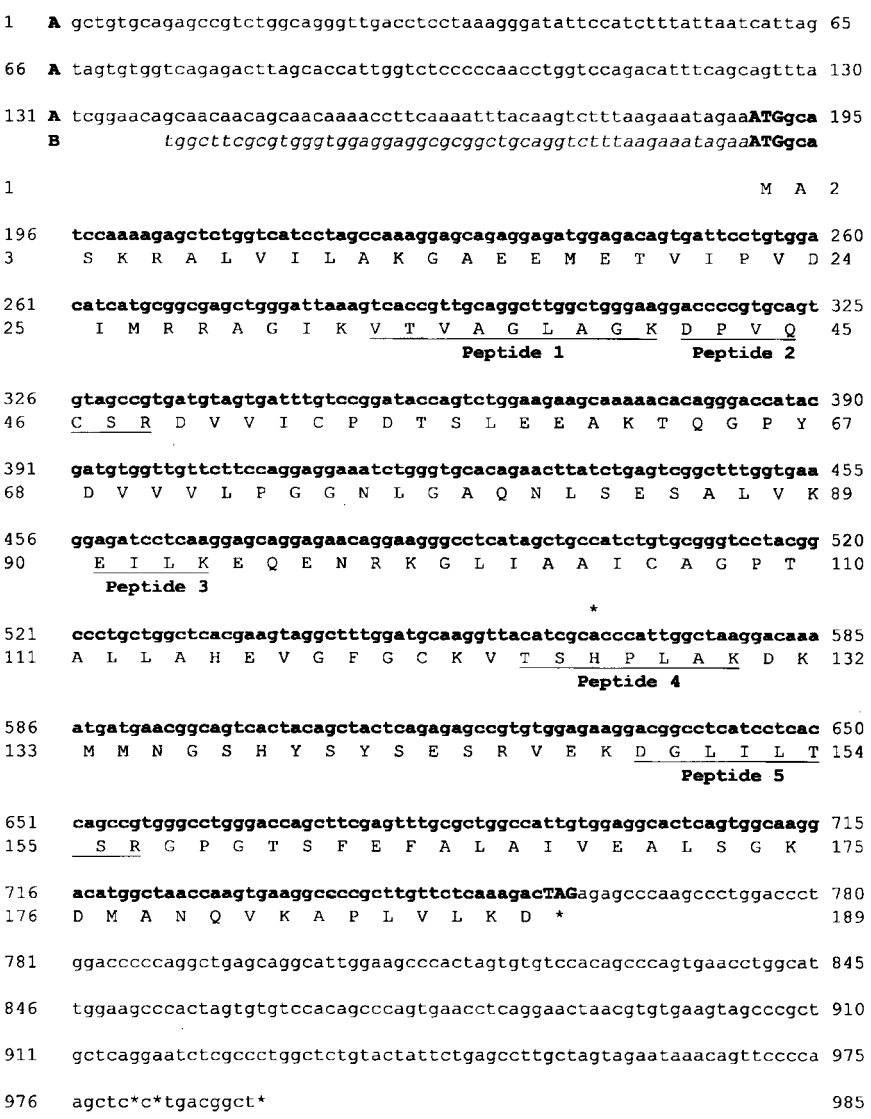

Figure 13. SP22 is encoded by alternatively-spliced mRNAs. The divergent 5' ends of two SP22 cDNAs designated A (plain text) and B (italics) are shown with the remaining sequence representing nucleotides conserved between these sequences. Putative coding sequences are shown in bold. Amino acid sequences derived from sequencing SP22 peptides are underlined. Peptide sequencing results matched perfectly with the exception of peptide # 4 where a histidine (H-asterisk) is observed in place of glycine (G). The canonical polyadenylation signal (AATAAA) is underlined. Observed multiple polyadenylation sites are indicated by asterisks.

Northern blotting with a probe derived from the region common to the SP22A and SP22B transcripts detected a 1.0 kb RNA in all somatic tissues examined (Figure 14). However, RNA from adult rat testis showed the presence of two transcripts at 1.0 and 1.5 kb respectively. To distinguish between the SP22A and SP22B transcripts, RNA from the developing rat testis was run on Northern blots and hybridized with transcript-specific probes derived from the unique 5' untranslated region (UTR) of either SP22A or SP22B. While the SP22B-specific probe hybridized with a single 1.0 kb transcript in all stages of testis development, the SP22A-specific probe first detected a 1.4-1.5 kb MRNA in day 15 testis RNA samples coincident with the initial differentiation of early spermatocytes. The level of the SP22A transcript

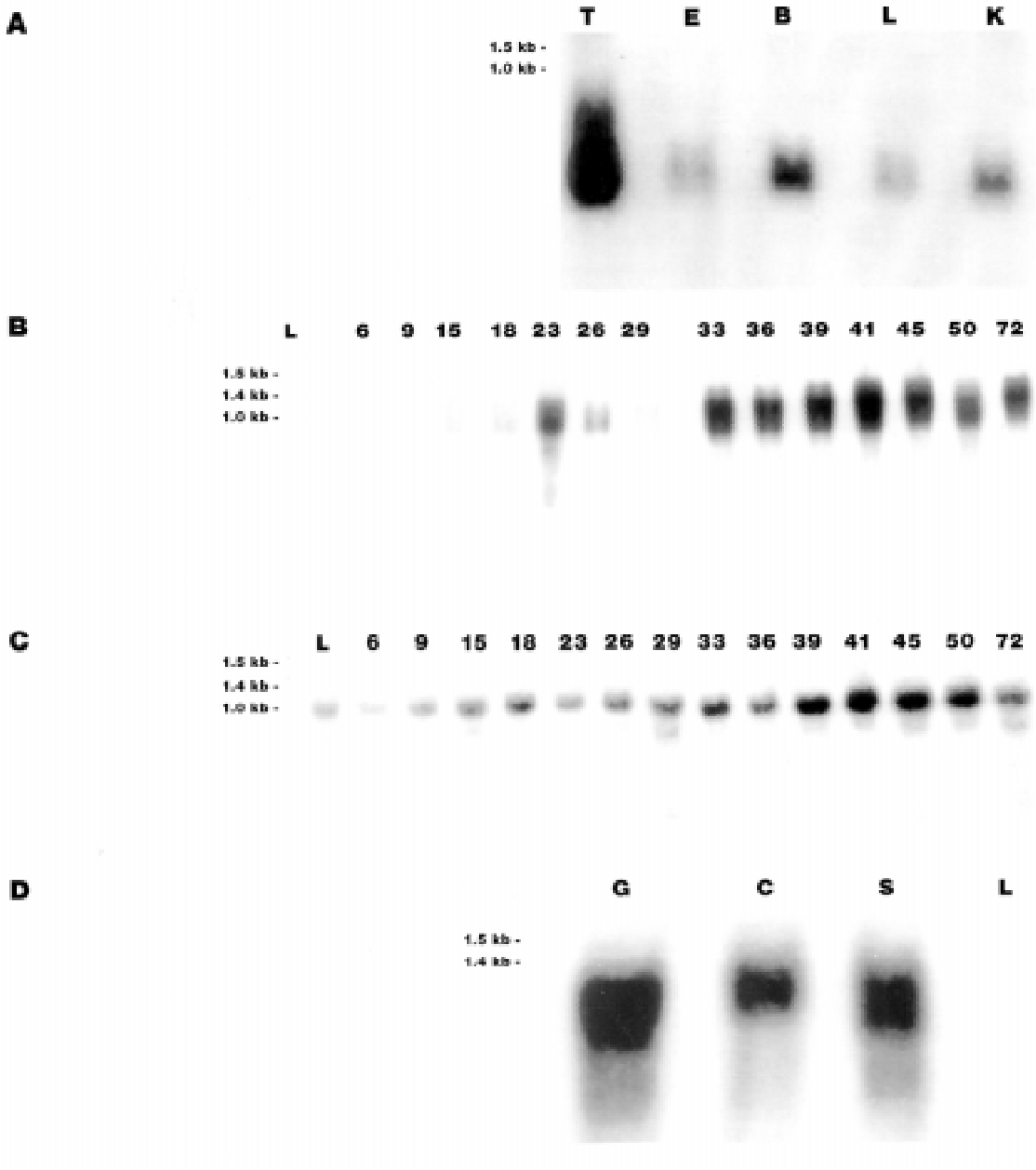

Figure 14. Transcription of the SP22 gene in rat somatic and reproductive tissues.

A) Using transcript-specific probes derived from the 5' UTR, the SP22A clone was shown to account for the 1.4-1.5 kb mRNA in the developing testis, but was not detectable in liver (L). Days of age are indicated over each testis lane. SP22A is developmentally regulated and was first detected in day 15 testis, coincident with the differentiation of early spermatocytes. The levels of SP22A mRNA increase slowly through day 29 (the day 23 lane was mistakenly overloaded) and then dramatically increase by day 33 as round spermatid develop suggesting that SP22A is expressed in these differentiating spermatogenic cells.

B) Northern blotting with a SP22B-specific probe detected a 1.0 kb transcript in liver and in all stages of testis development indicating that the SP22B mRNA is broadly expressed in the body.

C) To confirm that SP22A is expressed in spermatogenic cells, RNA was purified from mixed germ cells (G), pachytene spermatocytes (C), round spermatids (S), and Leydig cells (L) for Northern blotting with the SP22A-specific probe (fig. 4D). The 1.4-1.5 kb SP22 transcript was detected in all germ cell RNAs, but not in Leydig cells suggesting that SP22A expression might be confined to spermatogenic cells.

D) Northern blotting with a probe common to both SP22 transcripts detected a 1.0 kb mRNA in epididymis (E), brain (B), liver (L) and kidney (K) while the testis (T) was found to also express a larger ~1.5 kb transcript.

increased slowly through day 29 but became much more abundant by day 33, coinciding with the appearance of round spermatids. To confirm the presence of SP22A mRNA in male germ cells, various spermatogenic cell types and Leydig cells were isolated by centrifugal elutriation and Percall centrifugation as modified from a Leydig cell isolation protocol (Klinefelter et al., 1987); total RNA from the various germ cell fractions was purified for Northern blotting. The SP22A probe detected a 1.4-1.5 kb mRNA in a mixed population of spermatogenic cells, as well as in isolated pachytene spermatocytes and isolated round spermatids. No SP22A transcripts were seen in Leydig cell RNA. While these data do not rule out the expression of the larger SP22A transcript in Sertoli cells or other somatic cells in the testis, the larger SP22A mRNA is clearly developmentally regulated and is expressed in the differentiating spermatocytes and spermatids.

The presence of germ cell-specific transcripts of genes also transcribed in somatic cells is a well documented occurrence in the testis (Eddy et al., 1993). One biological reason associated with this phenomena is related to the changes occurring in DNA structure during the later stages of spermatogenic cell differentiation. While gene transcription occurs in developing gametes during the meiotic and early post-meiotic stages of spermatogenesis, the process of nuclear compaction mediated by the replacement of histones, first by transition proteins and subsequently by protamines, results in the transcriptional inactivation of the nucleus (Kierszenbaum & Tres, 1975). Therefore any protein synthesis needed during this period in development must come from mRNA transcribed earlier in differentiation. Spermatogenic cell-specific transcripts often have unique 5' or 3' UTR elements which confer increased message stability and allow these mRNAs to persist into later stages of post-meiotic haploid germ cell development (Penttila et al., 1995; Fajardo et al., 1997). The acquisition of a new 5'UTR in SP22A mRNA may serve to confer increased stability and could allow the translation of the SP22 protein in condensing spermatids.

The timing of protein synthesis in spermatogenic cells may also have an effect on the subcellular localization of the nascent protein. In transgenic mice expressing the human growth hormone (hGH) gene under the control of the protamine 1 promoter, translation during the early spermatid stage resulted in transport of the hGH protein to the acrosome (Braun et al., 1989). However, when the transgene is translationally regulated and protein first synthesized in elongating spermatids, hGH was localized to the cytoplasm. These results suggest that the timing of protein synthesis in spermatogenic cells can also control the localization of the protein and may result in a compartmentalization of SP22 in spermatozoa that is different from the distribution seen in somatic cells. Additionally, alterations in the length of the poly(A) tail appear to be related to the stability and translational activity of some germ cell mRNAs in late spermatogenesis with shortening of the poly(A) tracts

correlating with translational activity. Preliminary evidence suggests that SP22A may undergo some shortening of the poly(A) tail since treatment with oligo(dT) and RNAse H to remove the poly(A) tail collapses the 1.4-1.5 SP22A mRNAs into a single band.

As discussed previously, the peptide sequences obtained from SP22 matched a human protein termed DJ-1. Sequence alignments indicated a 90% identity with DJ-1 (Figure 15) and demonstrated that this protein represented a human version of SP22. Subsequent to the identification of SP22 and DJ-1, two additional reports appeared describing homologues of SP22 in rats and humans. Using an experimental protocol to test for epididymal toxicity that is similar to the one we have described herein, Contraceptive Associated Protein 1 (CAP1; Wagenfeld et al., 1998[a,b]) was identified as being homologous to DJ-1 and its cDNA was found to encode a protein identical with SP22. CAP1 was described as a rat epididymal sperm surface protein that is shed after treatments with ornidazole and alpha-chlorohydrin resulting in infertility; no functional studies have been reported. It is interesting to note that alignments of the

Figure 15. SP22 is a member of a highly conserved gene family. The most recent searches of the GenBank database found several characterized sequences with homology to the SP22 protein. The rat Contraceptive Associated Protein 1 (CAP1) was found to be a perfect match with SP22, while the human protein encoded by the DJ-1/RNA binding protein regulatory subunit (RS) sequences was 90% identical to rat SP22. An *Escherichia coli* protein designated thiJ exhibited a 40% amino acid identity, demonstrating that SP22 belongs to a highly conserved family of proteins. Dashs indicate identity, a colon indicates high similarity, and a single dot indicates weak similarity between residues.

SP22A nucleotide sequence with that of CAP1 indicate gaps of 50 nt and 42 nt in the 5' UTR and 3' UTR sequence of CAPI. It is unclear if this represents an alternative splicing of these regions or a cloning artifact, but the 3' UTR also contains 90 nt from a hepatome derived growth factor transcript and no additional SP22 clones exhibit the deletions seen in CAP1.

A second report describes a protein present in both human and rat cells which is postulated to function as a regulatory subunit of a RNA binding complex (Hod et al, 1999). While no sequence information was provided for the rat gene, the human sequence was identical to human DJ-1 and 90% identical to rat SP22. The preliminary functional description of the human SP22 homologue and their reported intracellular localization, which varied from a nuclear staining to a cytoplasmic distribution in a cell dependent manner, is difficult to reconcile with the surface localization of SP22 on spermatozoa and its appearance in epididymal fluid after toxicant exposure. However, all immunolocalization data was obtained from transformed cells and not normal tissues. This implies that SP22 does not reach the cell surface in transformed cells in culture as it does in spermatozoa and suggests that SP22 may function differently in transformed cells than it does in the male gamete.

More extensive searching of DNA sequence databases indicate SP22 is a member of a highly conserved gene family present in organisms as divergent as Escherichia coli. The E. coli homologue of SP22 was designated thiJ for its postulated involvement as a 4methyl-5(p-hydroxyethyl)-thiazole monophosphate biosynthetic protein in the synthesis of thiamine, vitamin Bl (Backstrom, 1996). However, subsequent studies revealed that another gene was responsible for this step in thiamine synthesis and no function is currently known for thiJ. The thiJ protein is almost exactly the same size and exhibits a 40 % identity with SP22. Mammalian sequence homologues to SP22 have now been identified from the GenBank database in human, mouse, rabbit, and pig (Figure 16). Genes from organisms as diverse as *Salmonella bacteria, Borrelia spirochetes, Mesembryanthemum* ice plants, and *Caenorhabditis elegans* nematodes that show homology with SP22 also remain functionally uncharacterized.

SP22's Role - Coincidental or Causal?

Realizing that it was possible that the correlation between SP22 levels and fertility was coincidental, we next sought to determine whether SP22 plays a functional role in the process of fertilization. When affinity-purified SP22 peptide antibody was incubated with proximal cauda sperm for 5 minutes prior to in utero insemination, fertility was markedly reduced (data not shown); in fact only one of six inseminated females had any implants on gestation day 9 and the number of implants in this female was below control levels. While these data were suggestive of a functional

role, it was concluded that a second, in vitro experiment was necessary to control for any agglutination of sperm that might occur in the presence of the antibody and thereby confound interpretation of the fertility data. Indeed, when antibody was added to sperm prior to in vitro insemination of rat eggs a significant degree of agglutination was evident. However, when Fab fragments of the antibody were generated, agglutination was minimal and a significant reduction in fertilization rate was still observed (Klinefelter et al., 1999). Indeed, using Fab (1: 100) the fertilization rate declined from 68 % to 9 % (Figure 17A). Importantly, when SP22 peptide was added to the Fab at the onset of incubation, fertilization rate was unaffected.

```
            1                                                    50
   HUMAN    MASKRALVIL AKGAEEMETV IPVDVMRRAG IKVTVAGLAG KDPVQCSRDV
   MOUSE    MASKRALVIL AKGAEEMETV IPVDVMRRAG IKVTVAGLAG KDPVQCSRDV
     PIG    .......... .......... .......... .......... ..........
  RABBIT    MASKRALVIL AKGAEEMETV IPVDVMRRAG IKVTVAGLAG KDPVQCSRDV
     RAT    MASKRALVIL AKGAEEMETV IPVDIMRRAG IKVTVAGLAG KDPVQCSRDV

IDENTITY    MASKRALVIL AKGAEEMETV IPVD.MRRAG IKVTVAGLAG KDPVQCSRDV

            51                                                  100
   HUMAN    VICPDASLED AKKEGPYDVV VLPGGNLGAQ NLSESAAVKE ILKEQENRKG
   MOUSE    MICPDTSLED AKTQGPYDVV VLPGGNLGAQ NLSESPMVKE ILKEQESRKG
     PIG    .......... .......... .......... NLSESAAVKD ILKEQEKRKG
  RABBIT    VICPDAXLED AKKEGPYDVV VLPGGNLGAQ NLSESAAVKE ILKEQEKXKG
     RAT    VICPDTSLEE AKTQGPYDVV VLPGGNLGAQ NLSESALVKE ILKEQENRKG

IDENTITY    .ICPD.SLE. AK..GPYDVV VLPGGNLGAQ NLSES..VK. ILKEQE.RKG

            101                                                 150
   HUMAN    LIAAICAGPT ALLAHEIGCG SKVTTHPLAK DKMMNGGHYT YSENRVEKDG
   MOUSE    LIAAICAGPT ALLAHEVGFG CKVTTHPLAK DKMMNGSHYS YSESRVEKDG
     PIG    LIAAICAGPT ALLAHEIGFG SKVTTHPLAK DKMMNGSHYS YSENRVEKDG
  RABBIT    LIAAICAGPT ALLXHEIGFG SKVTHHPLAK DKMMNG.... ..........
     RAT    LIAAICAGPT ALLAHEVGFG CKVTSHPLAK DKMMNGSHYS YSESRVEKDG

IDENTITY    LIAAICAGPT ALLAHE.G.G .KVT.HPLAK DKMMNGSHY. YSE.RVEKDG

            151                                          189
   HUMAN    LILTSRGPGT SFEFALAIVE ALNGKEVAAQ VKAPLVLKD
   MOUSE    LILTSRGPGT SFEFALAIVE ALVGKDMANQ VKAPLVLKD
     PIG    LILTSRGPGT SFEFALAIVE ALAGKEVADQ VKAPLVLRD
  RABBIT    .......... .......... .......... .........
     RAT    LILTSRGPGT SFEFALAIVE ALSGKDMANQ VKAPLVLKD

IDENTITY    LILTSRGPGT SFEFALAIVE AL.GK..A.Q VKAPLVL.D
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Figure 16. SP22-like sequences are expressed in a large number of mammalian species. SP22-like sequences were identified in human, mouse, pig, and rabbit and used for comparison with rat SP22. The resulting consensus sequence exhibited an 89% identity among the five sequences and suggests that SP22 is highly conserved among mammalian species.

While the weight of *in vitro* evidence strongly suggested that SP22 played a pivotal role in fertilization, it was deemed important to return to in utero insemination using the Fab preparation. When proximal cauda epididymal sperm were incubated with Fab (1:50) for 5 minutes prior to in utero insemination, the fertility observed on gestation day 9 was significantly reduced from control values of 82 % to only 45 % (Figure 17B). When SP22 peptide was added to the sperm-Fab incubation prior to insemination, the resultant fertility was not significantly different from control values; 77 % vs 82 %. These data demonstrate convincingly that SP22 does indeed play a causal role in the process of fertilization.

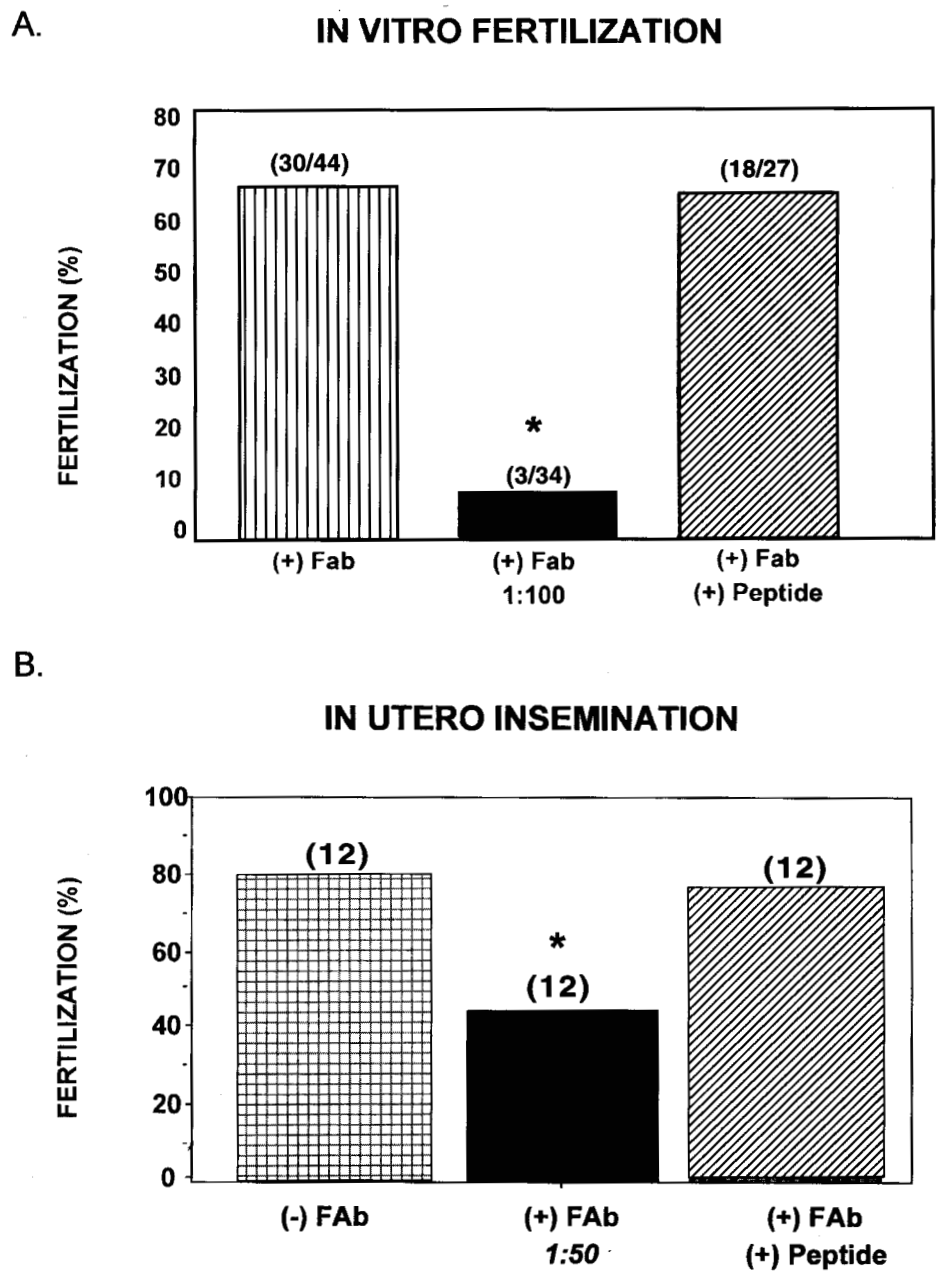

Figure 17. A) The rat in vitro fertilization rate, expressed as the percentage of eggs ( ) containing a sperm tail. When cauda epididymal sperm were incubated overnight with eggs in the presence an Fab preparation (1:100) of the affinity-purified anti-SP22 peptide antibody, the fertilization rate was decreased significantly. **B)** When cauda epididymal sperm were incubated briefly (5 min) with Fab (1:50) prior to in utero insemination, fertility (implants/ corpora lutea on gestation day 9) was significantly decreased. Addition of SP22 peptide to the sperm-Fab mixture restored fertility to a control level (N= number of males).

Generation and Utility of Recombinant SP22

Elucidation of SP22 function in spermatozoa requires a sufficiently large amount of purified SP22 protein. For this, a recombinant SP22 expression cassette encoding the entire SP22 protein was synthesized by PCR amplification of the coding region from the SP22 cDNA. This cassette was cloned into a pQE8 plasmid containing a prokaryotic lac promoter region and the Shine-Delgarno ribosome binding site to facilitate expression in E. coli. The plasmid also contained a sequence encoding a series of six histidine residues (6xHis) to allow rapid purification of the recombinant protein (Berthhold et al., 1992). Once transfected into E. coli, SP22 production was induced by the addition of isopropyl-thio-b-D-galactopyranoside (IPTG) to activate the lac promoter. The 6xHis tag has a pH-dependent high affinity for nickel and is capable of binding in the presence of high concentration of urea and guanidinium salts. After growth in culture for 5-6 hours, cells were harvested by centrifugation and solubilized in 6 M guanidine hydrochloride, 100 mM phosphate at pH 8.0. The nickel agarose column was washed stepwise in 8M urea, 100 mM phosphate buffer at pH 8.0, pH 6.3, pH 5.9, and finally pH 4.5. The bacterial proteins were either not retained on the column, or were eluted in the first three washes, while recombinant SP22 (rSP22) was eluted at pH 4.5. The purified rSP22 yields per one liter of bacterial culture were in the milligram range.

The availability of rSP22 has already proven to be a valuable tool and has allowed the production of high titer polyclonal antisera against SP22. The rSP22 is a full length recombinant as its migration in a 14 % gel is identical to that of SP22 in detergent extracts of sperm, i.e. 28 kD. Moreover, this recombinant presents all of the charged variants that are seen using the anti-peptide antibody to SP22 (Figure 18). Immunostaining using an affinity-purified polyclonal antibody to rSP22 reveals an expanded localization of SP22 relative to earlier immunostaining with SP22 peptide antibody (Figure 19). Fluorescence is evident over the entire length of the equatorial segment of the rat sperm head, rather than just over the anterior ventral segment as observed using the anti-peptide antibody. Likewise, a discrete equatorial staining pattern is evident over bull and human sperm. Sperm recovered from the proximal cauda epididymidis 3 days after castration express little SP22, but when rSP22 is added prior to insemination, SP22 detection is significantly enhanced (Figure 20). Thus, rSP22 and anti-rSP22 can now being used in conjunction with in utero insemination and in vitro fertilization to access SP22's role in fertility and specific facets of sperm-egg interaction. Finally, bacteria mutants missing the SP22 homologue (thiJ) can now be used to investigate protein function and the SP22 protein can be expressed as discrete domains for functional studies in both bacterial and mammalian systems.

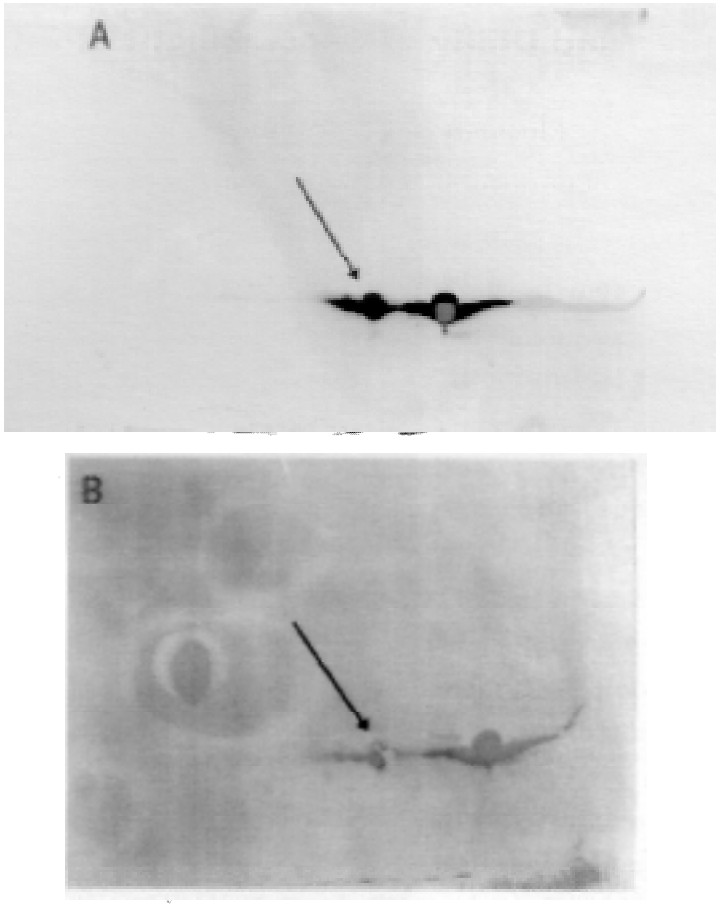

Figure 18. A) Silver-stained 2D SDS PAGE of recombinant SP22 (rSP22). Notice that SP22 (arrow) and its charged variants persist. **B)** A corresponding immunoblot demonstrating that SP22 and its charged variants are all recognized by affinity-purified anti-SP22 peptide antibody.

SP22's Potential as a Biomarker of Fertility

Once we discovered that a unique SP22 transcript appears in the testis, and that sperm recovered from the rete testis immunostain for SP22, we explored the possibility that testicular toxicants could compromise the levels of SP22 on sperm, and that this might once again translate into reductions in fertility from these males. For this we elected to study bromochloroacetic acid, a prevalent byproduct of drinking water disinfection, and one of several di-substituted haloacids recently shown to produce spermatotoxicity in the rat testis (Linder et al, 1995, 1997; Strader et al. 1998). Interestingly the di-substituted haloacids, particularly the brominated species, disrupt the latter stages of spermatogenesis, i.e. spermiogenesis. In this regard one typically finds retention of step 19 spermatids in Stages IX and X of the spermatogenic cycle, the formation of atypical residual bodies, spermatids sharing a common acrosome, and mature sperm that are actually fused. Collectively, these toxicant-induced lesions imply that Sertoli cell-spermatid interactions are compromised. Since, normal Sertoli cell - spermatid interactions are pivotal to successful spermatid maturation , we hypothesized that qualitative changes would also be occurring to the developing sperm membrane as a result of toxicant exposure, and that both SP22 and fertility would in turn be affected.

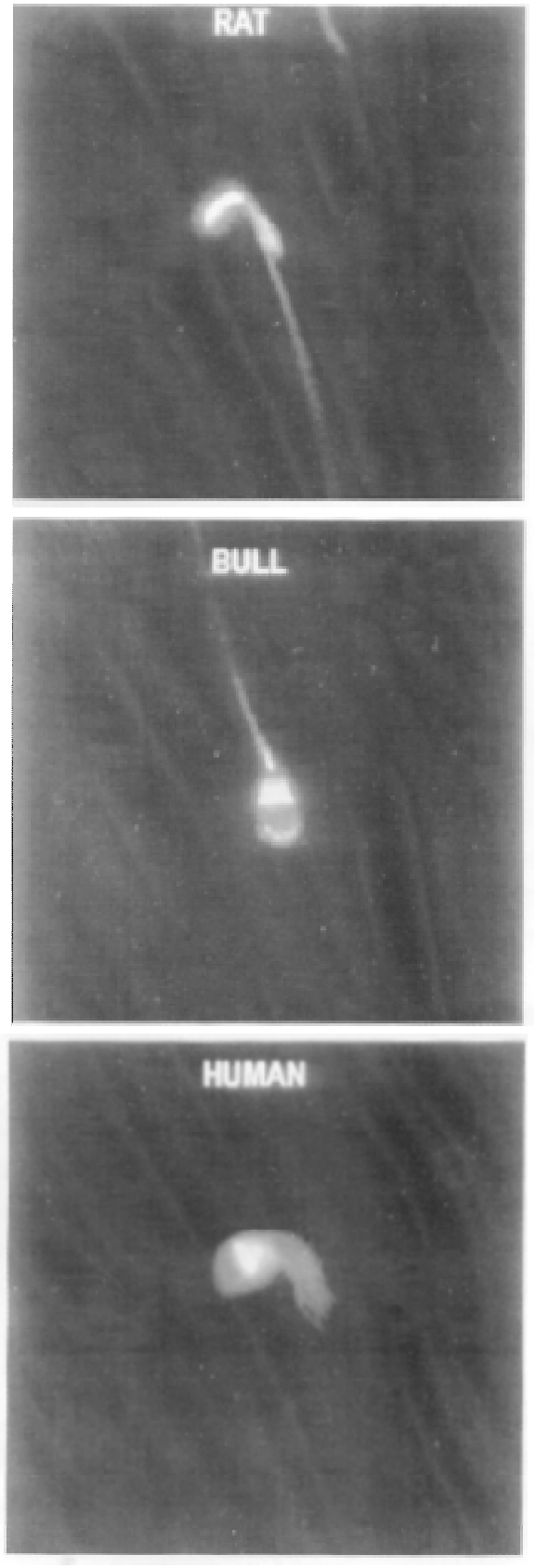

Figure 19. Light micrographs showing immunofluorescent staining of sperm labeled with affinity purified antibody to rSP22. Notice that the staining over the rat head now extends beyond the anterior-ventral surface to include the entire equatorial region. The staining on the bull and human sperm is also consistent with equatorial localization.

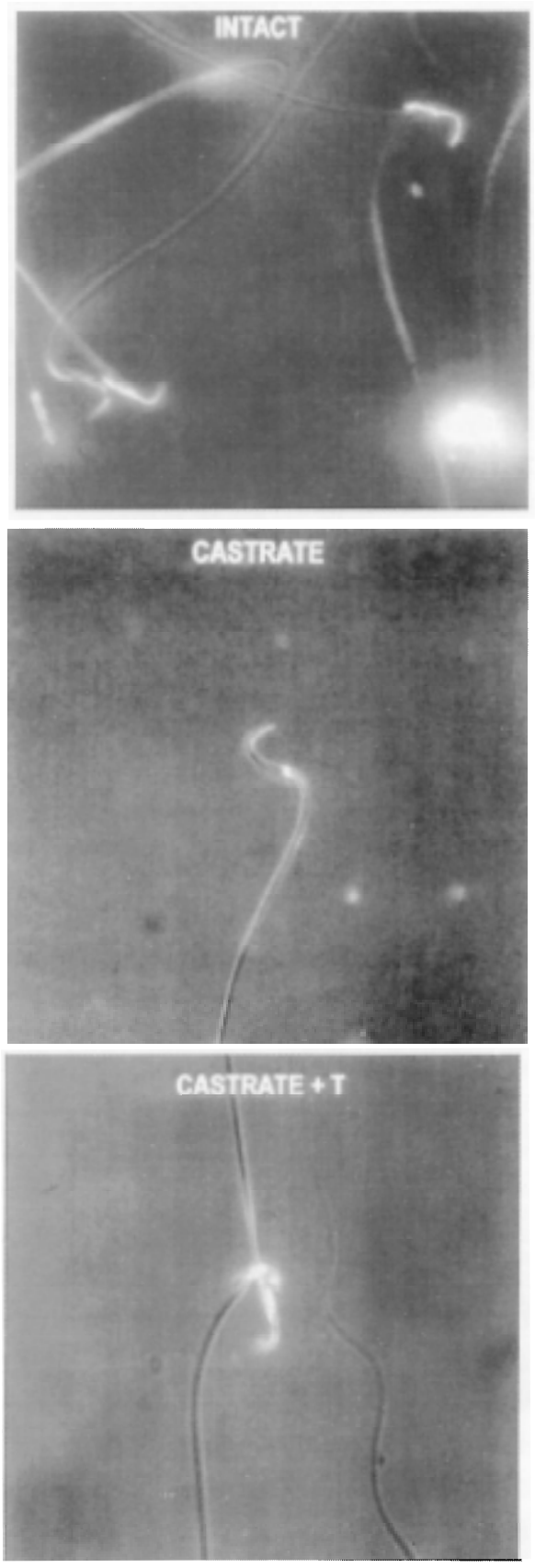

Figure 20. Light micrographs showing immunofluorescent staining of rat sperm labeled with affinity purified antibody to rSP22. Notice that the staining is greatly diminished on sperm from the proximal cauda epididymidis 3 days post-castration. By contrast, a brief 5 minute incubation with rSP22 just prior to in utero insemination results in significantly enhanced localization over the surface of the head.

For this study animals were dosed daily for 14 days to implicate the developing spermatids at the onset of the exposure. After these two weeks of dosing, the animals were terminated, and the proximal cauda sperm were recovered and used for in utero insemination and protein quantification as in our previous study. Both fertility and the background-corrected integrated optical density of SP22 in 2D gels of detergent sperm extracts were decreased significantly at each dosage of bromochloroacetic acid tested (data not shown). The fact that the magnitude of the decrease in both fertility and SP22 level was consistent within each dose suggested that these endpoints were once again correlated with one another. Indeed, when the data for these two endpoints were fit to the same nonlinear equation used in our previous study, the derived correlation (r^2) was 0.84. Moreover, if the data for fertility and SP22 from both of our studies (i.e. the epididymal toxicant study and the bromochloroacetic acid study) are fit collectively to this equation and analyzed, the resultant correlation is 0. 82 (Figure 21). These results strongly suggest that whether SP22 levels on sperm are compromised by a testicular or epididymal insult: 1) the diminution in SP22 is highly-correlated with the resultant fertility, and 2) a non-linear (i.e. threshold) relationship appears to exist between the SP22 levels on sperm and the fertility of these sperm. By determining a threshold value for SP22 based on the point on the curve where the confidence interval is the narrowest (i. e. where the confidence level is greatest), the overall predictability of fertility can be evaluated. From the figure it is obvious that at the SP22 threshold, fertility equals approximately 50 %. Moreover, 90 % of the animals with sperm SP22 levels greater than this threshold were classified by discriminant analysis as fertile (i. e. greater than 50% fertility) and 82% of the animals with SP22 levels less than this threshold were classified as subfertile (i.e. less than 50% fertility). Thus, it seems reasonable to hypothesize that the fertility of a population of sperm might be predicted on the basis of the level of SP22 expression.

While the correlation between fertility and detergent-extracted SP22 quantified in 2D gels has been established, quantification of 2D gel proteins is time consuming and tedious. We have now evaluated sperm from a small number of bulls with varying relative breeding efficiencies using FITC staining of bound affinity-purified anti-rSP22 antibody as a indicator of SP22 expression rather than quantification of detergent-extracted SP22 in 2 D gels. There were marked differences in the level of FITC expression between bulls (Figure 22 A), and while the data are not robust enough for fitting to our threshold model, the scatter plot does indeed suggest that bulls with higher relative breeding efficiencies tend to have a higher level of SP22 expression (Figure 22B). Current efforts are now underway to apply a diagnostic immunoassay to sperm from animals in toxicology studies as well as sperm from men in epidemiologic field studies.

The concept of a diagnostic assay for the fertility of sperm based on the expression of a specific sperm protein is not novel. Indeed, both SP10 (Herr and Wright, 1997) and P34H (Sullivan, 1998) have been proposed for use as a human diagnostic of male fertility. SP10 represents a series of 18-34 kD proteins localized on the

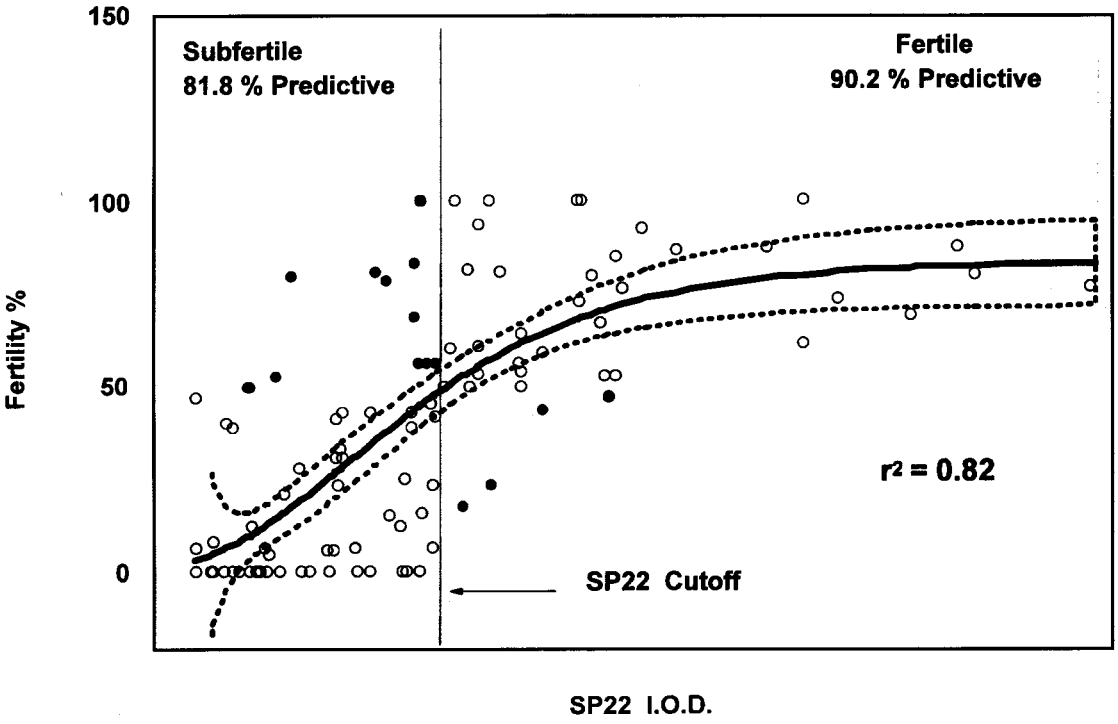

Figure 21. Scatter plot depicting the relationship between fertility and sperm SP22 levels from all animals (N=107) in both the epididymal toxicity and bromochloroacetic acid studies. Data were fit to the same non-linear, threshold equation used in the initial study and the resultant r^2 = 0.82. Based on the confidence interval (dashed lines) we have selected a cutoff for SP22 threshold (solid vertical line). At this threshold SP22 value, fertility is approximately 50%. Assuming that animals with fertility greater than this predicted value are fertile, 90.2 % of the animals in the data set were correctly classified, suggesting that this model is at least 90 % predictive of fertility. Likewise, this model indicates an 82 % predictive value for identifying a subfertile animal (i.e. an animal with fertility less than 50 %). Solid circles represent animals that were not accurately classified.

luminal surface of the inner and outer acrosomal membranes (Herr et al., 1992). Once antibodies to human SP10 were observed to inhibit the binding of capacitated sperm to the zona pellucida (Coonrod et al., 1996), this protein was thought to have potential as both a diagnostic and a contraceptive antigen. The human sperm surface protein P34H is homologous to the P26h hamster antigen (Boue et al., 1994). This protein is localized over the acrosome and is expressed initially in the proximal regions of the epididymis. That its expression intensifies after capacitation and then disappears following the acrosome reaction argues for a role in sperm egg binding (Boue et al., 1996) and prompted its consideration as a diagnostic. Unlike these proteins which have ignited interest based on results obtained with capacitated sperm, SP22 has been linked to fertility based on its expression on cauda epididymal or ejaculated sperm, i.e. prior to capacitation. This may be an important consideration in the development of a diagnostic immunoassay, particularly with regard to the speed and ease with which data might be obtained. Moreover, it is at least reasonable to assume that the more proximate biomarkers or indicators of the fertility of sperm might play important roles prior to capacitation. In this regard, one could envision that capacitation and the expression of capacitation-linked antigens might not occur if

the expression of proteins such as SP22 is not favorable. However, a role such as this would not preclude a role at the site of fertilization as well. Indeed, the fact that there are multiple testicular transcripts for SP22 suggests that there may well be multiple forms of SP22 expressed on sperm. These isoforms may manifest at different times throughout the process of fertilization (i.e. in situ or upon ejaculation, capacitation, egg binding, and acrosome reaction, etc.), with different patterns of expression (i.e. acrosomal, equatorial, etc.), and with different molecular functions (i.e. epididymal maturation, capacitation, sperm-egg binding, etc.).

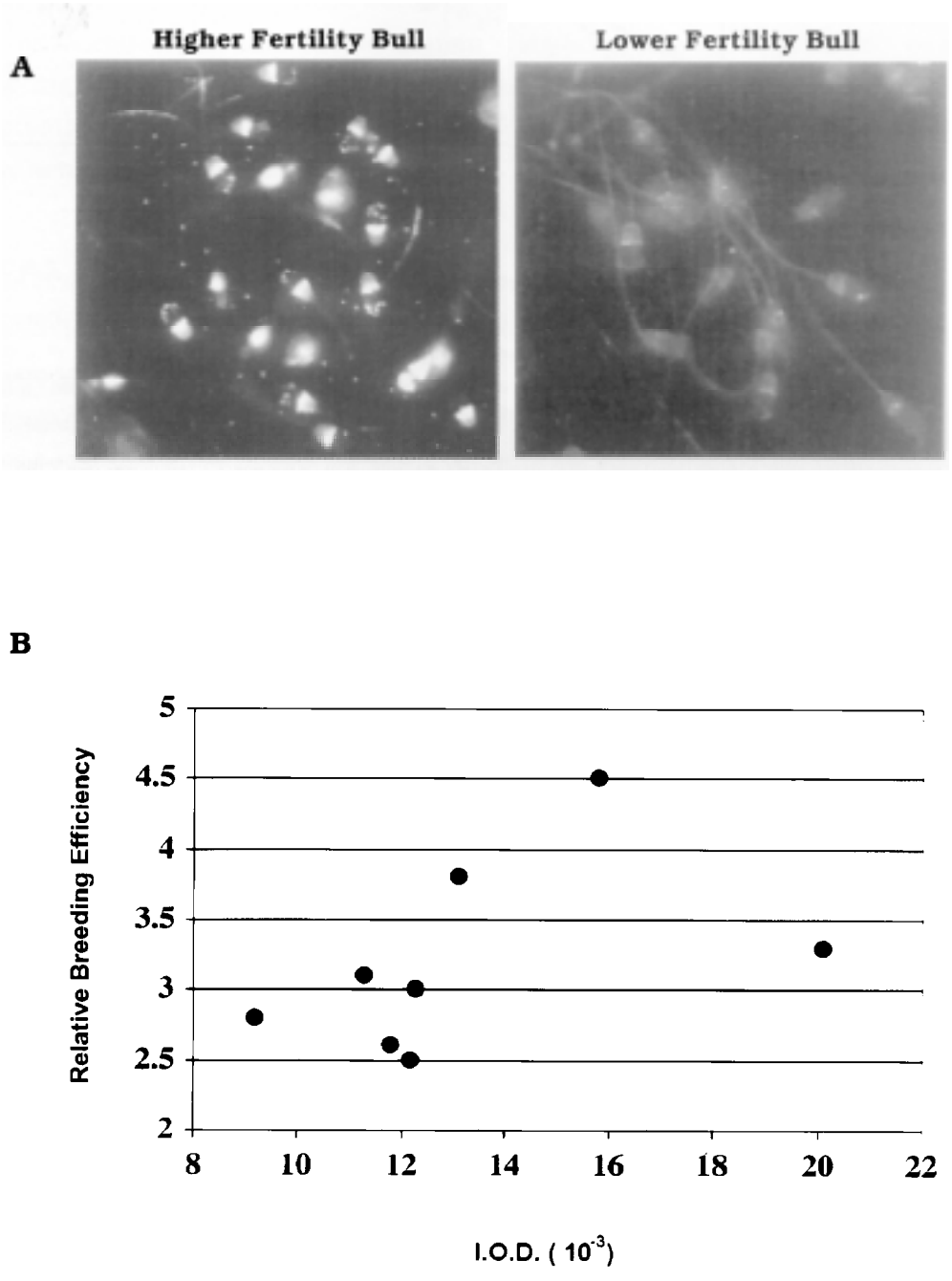

Figure 22. A) Micrographs depicting FITC localization on bull sperm using the affinity purified anti-rSP22 peptide antibody. There is enhanced staining over the equatorial segment on sperm from the bull with higher fertility compared to those from the bull with lower fertility. B) A scatter plot of the integrated optical density of the FITC signal over the equatorial segment (averaged for 200 sperm) and the relative breeding efficiency (RBE) of the bull from which these sperm were collected. In general bulls with an RBE greater than 3.0 are considered to be higher fertility. Notice that there is some trend between bulls with higher RBE's and their SP22 levels.

Is SP22 a Candidate for Therapeutic Enhancement or Contraception?

In recent years there has been some effort to demonstrate that the exogenous administration of specific protein can actually enhance the fertilizing potential of sperm (Amann et al, 1999). This work demonstrated that when 30 uM concentrations of a 60 amino acid peptide of the Sertoli cell protein Prosaposin, commonly referred to as SGP-1 (Collard et al., 1988) were added to bull sperm prior to artificial insemination, fertility was enhanced significantly. While neither the biological role of SGP-1 on sperm , or the mechanism underlying SGP-1 enhancement of fertility are understood, this observation raises the intriguing possibility that other, and even more definitive fertility-associated proteins, might also be capable of enhancing the fertility of sperm. To this end, we have now initiated experiments to determine whether exogenous addition of SP22 can enhance the fertility of cauda epididymal sperm in rats. For this, we have implemented two different experimental models. In one model we add recombinant SP22 to proximal cauda epididymal sperm from rats 2-3 days post-castration and incubate briefly prior to in utero insemination. The other involves the addition of recombinant SP22 to a suboptimal number (i.e. less than 5 x 10^6 per uterine horn) of proximal cauda epididymal sperm prior to in utero insemination. From the immunostaining with antibody to recombinant SP22 (above), It appears that the addition of recombinant SP22 is increasing the level of SP22 on the sperm surface. While data are preliminary, it appears that addition of recombinant SP22 can improve the fertility of sperm that have either been compromised in vivo, or sperm that are "normal" but are inseminated at a sufficiently low dosage to facilitate enhancement. Finally, we have recently succeeded in the purification of native SP22, and it is possible that the exogenous addition of native SP22 will intercalate into the plasma membrane more effectively which may or may not result in quarter enhancement of fertility. Alternatively, once functional domains of SP22 are characterized small molecular agonists may be developed.

Just as numerous sperm proteins have been identified as prospective diagnostic indicators of fertility, sperm proteins have been considered as putative contraceptive vaccines. Of these, the most notable are PH-20 (Primakoff et al., 1988, 1997[a,b]), Fertilin (Ramarao et al., 1996; Cho et al., 1998), SP-10 (Coonrod et al., 1996; Herr et al., 1997), SP17 (O'Rand & Widgren, 1994; O'Rand et al., 1996), Protein D/E (Ellerman et al., 1998) and LDH-C4 (Goldberg et al., 1981; O'Hern et al., 1995). While most of these candidate vaccines have successfully produced infertility, the results obtained with PH-20 (Primakoff et al., 1997) and LDH-C4 (O'Hern et al., 1995) are among the most intriquing to date as immunization of animals has resulted in nearly complete infertility and subsequent reversibility. However, even in these cases, infertility following immunization is not guaranteed, and more importantly, the targeted effect(s) of the immunization have not yet been clearly established. While SP22 is a surface antigen on sperm, the protein is ubiquitously expressed throughout the body and immunization is therefore likely to result in profound extragonadal effects. To

circumvent this issue, we need to remain focused on identifying the functional role, and functional domains, of SP22 on spermatozoa. Once again, a viable alternative to immuno-contraception may be targeting SP22 through a pharmaceutical approach using small molecular antagonists to functionally disrupt the function of the protein. Alternately, the use of small recombinant peptides derived from specific domains of SP22 may also have contraceptive potential if they are designed to block the function of SP22, possibly by interfering with the interaction of SP22 with either sperm or oocyte proteins. Such small peptides are often less antigenic and may not result in an immune response, particularly if not administered systemically. Finally, since a decrease in SP22 levels on spermatozoa are correlated with a reduction in fertility after chemical exposure, compounds which potentiate the loss of SP22 during transit through the reproductive tract would be expected to diminish fertility and may have a contraceptive potential. While human contraceptives are required to work with very high efficiency, it should be remembered that in other situations, including population control of rapidly breeding wildlife species, a 50% reduction in fertility may achieve the desired result.

References

Amann RP. The use of animal models for detecting specific alterations in reproduction. Fundam Appld Toxicol 1986;2:13-26.

Amann RP, Hammerstedt RH, Veeramachaneni DNR. The epididymis and sperm maturation: a perspective. Reprod Fertil Dev 1993;5:361-81.

Amann RP, Seidel GE, Brink ZA. Exposure of thawed frozen bull sperm to a synthetic peptide before artificial insemination increases fertility. J Androl 1999;20:42-6.

Backstrom AD. The biosynthesis of thiamin in Escherichia coli K-12: structural genes for thiamin biosynthetic enzymes (thiCEFGH and thij) and function of the thie gene product (thimine phosphate synthatse [E.C. 2.5.1.3]). Ithaca, New York: Cornell University, 1996. PhD Disseration.

Bedford JM. Maturation, transport, and fate of spermatozoa in the epididymis. In: Hamilton DW, Greep RO, eds. Handbook of phsyiology, male reproductive system. Washington DC: American Physiological Society, 1975;303-18.

Boue F, Berube B, de Lamirande E, Gagnon C, Sullivan R. Human sperm zona pellucida interaction is inhibited by an antiserum against a hamster sperm protein. Biol Reprod 1994;51:577-87.

Boue F, Sullivan R. Cases of human infertility are associated with the absence of P34H, an epididymal sperm antigen. Biol Reprod 1996;54:1018-24.

Braun RE, Peschon JJ, Behringer RR, Brinster RL, Palmiter RD. Protamine 3'untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. Genes Dev 1989;3:793-802.

Brooks DE, Higgins SJ. Characterization and androgen-dependence of proteins associated with luminal fluid and spermatozoa in rat epididymis. J Reprod Fert. 1980;59:363-75.

Burks DJ, Carballada R, Moore HDM, Saling PM. Interaction of a tyrosine kinase from human sperm with the zona pellucida at fertilization. Science 1995;269:83-9.

Cho C, Bunch D, Faure J-E, Goulding EH, Eddy EM, Primakoff P, Myles DG. Fertilization defects in sperm from mice lacking Fertilin B. Science 1998;281:1857-9.

Chodavarapu SR, Myles DG, White JM, Priinakoff P. Initial evaluation of Fertilin as an immunocontraceptive antigen and molecular cloning of the cynomolgus monkey Fertilin B subunit. Mol Reprod Develop 1996;43:70-5.

Cohen DJ, Munuce MJ, Cuasnicu PS. Mammalian sperm-egg fusion: the development of rat oolemma fusibility during oogenesis involves the appearance of binding sites for sperm protein DE. Biol Reprod 1996;55:200-206.

Collard MW, Sylvester SR, Tsuruta JK, Griswold MD. Biosynthesis and molecular cloning of sulfated glycoprotein-1 secreted by rat Sertoli cells: sequence similarity with the 70-kilodalton precursor to sulfatide/GM 1 activator. Biochemistry 1988;27:4557-64.

Comhaire F, Van Waeleghem K, De Clercq N, Schoohjans F. Declining sperm quality in European men. Andrologia 1996;28:300-1.

Comhaire F, Van Waeleghem K, De Clercq N, Vermeulen L, Schoonjans F. Statement on the general reduction in sperm quality. Int J Androl 1995;18:1-2.

Coonrod SA, Westusin ME, Herr JC. Inhibition of bovine fertilization in vitro by antibodies to SP-10. J Reprod Fert 1996;107:287-97.

Cuasnicu PS, Echeverria FG, Piazza, AD, Cameo MS, Blaquier JA. Antibodies against epididymal glycoproteins block fertilizing ability in rat. J Reprod Fert 1984;72:467-71.

Cuasnicu PS, Gonzalez EF, Piazza A, Blaquier, JA. Addition of androgens to cultured hamster epididymis increases zona recognition by immature spermatozoa. J Reprod Fert 1984;70:541-7.

Dyson AL, Orgebin-Crist M-C. Effect of hypophysectomy, castration, and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. Endocrinology 1973; 93:391-402.

Eddy EM, Welch JE, O'Brien DA. Gene expression during spermatogenesis. In: de Kretser DM, ed. The molecular biology of the male reproductive system. Orlando: Academic Press, 1993:181-232.

Ellerman DA, Brantúa VS, Martinez SP, Cohen DJ, Conesa D, Cuasnicu P. Potential contraceptive use of epididymal proteins: immunization of male rats with epididymal protein DE inhibits sperm fusion ability. Biol Reprod 1998;59:1029-36.

Fajardo MA, Butner KA, Lee K, Braun RE. Germ cell-specific proteins interact with the 3' untranslated regions of Prm-1 and Prm-2 MRNA. Dev Biol 1994;166:643-53.

Garberi JC, Kohane AC, Carneo MS, Blaquier JA. Isolation and characterization of specific rat epididymal proteins. Mol Cell End 1979;13:73-82.

Giwercman A, Skakkebaek NE. The human testis-an organ at risk? Int J Androl 1992;15:373-5.

Goldberg E, Gonzales-Prevatt V, Wheat TE. Immunosuppression of fertility in females by injection of sperm-specific LDH-C4 (LDH-X): prospects for development of a contraceptive vaccine. In: Senun K, Mettler L, eds. Human reproduction, proceedings of the IIIrd World Congress. Amsterdam: Excerpta Medica, 1981:360-3.

Herr JC, Klotx K, Shannon J, Wright RM, Flickinger CJ. Purification and microsequencing of the intra-acrosomal protein SP-10. Evidence that SP-10 heterogeneity results from endoproteolytic processes. Biol Reprod 1992;47:11-20.

Herr JC, Wright RM. Human sperm diagnostic. US Patent #231,675, 1997.

Herr JC, Wright RM. Primate intra-acrosomal sperm antigen for use in a contraceptive vaccine. US Patent #5,602,005, 1997.

Hod Y, Pentyala SN, Whyard, TC, El-Maghrabi, MR. Identification and characterization of a novel protein that regulates RNA-protein interaction. J Cell Biochem 1999;72:435-44.

Iglesias AA, Andreo CS. NADP-dependent malate dehydrogenase (decarboxylating) from sugar cane leaves. Kinetic properties of different oligomeric structures. Eur J Biochem 1990;192:729-33.

Jackson H, Fox BW, Craig AW. Antifertility substances and their assessment in the male rodent. J Reprod Fert 1961;2:447-65.

Jackson CM, Morris ID. Gonadotropin levels in male rats following impairment of Leydig cell function by ethylene dimethanesulphonate. Andrologia 1977;9:29-35.

Jackson H. The effects of alkylating agents on fertility. Brit Med Bull 1964;20:107-14.

Kaplan JM, Varmus HE, Bishop JM. The src protein contains multiple domains for specific attachment to membranes. Mol Cell Biol 1990;10:1000-9.

Killian G, Chapman D, Cancel A, Henault, MA. Alteration and prediction of male fertility using seminal plasma and its components. US Patent #5,569,581, 1996.

Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein. Bull seminal plasma. Biol Reprod 1993;49:1202-7.

Kierszenbaum AL, Tres LL. Structural and transcriptional features of the mouse spermatid genome. J Cell Biol 1975;65:258-70.

Klinefelter GR, Hamilton DW. Synthesis and secretion of proteins by perifused caput epididymal tubules, and association of secreted proteins with spermatozoa. Biol Reprod 1985;33:1017-27.

Klinefelter GR, Laskey JW, Perreault SD, Ferrel J, Jeffay S, Suarez J, Roberts N. The ethane dimethanesulphonate-induced decrease in the fertilizing ability of cauda epididymal sperm is independent of the testis. J Androl 1994;15:318-27.

Klinefelter GR, Laskey JW, Roberts NL, Slott V, Suarez JD. Multiple effects of ethane dimethanesulphonate on the epididymis of adult rats. Toxicol Appld Pharmacol 1990;105:271-87.

Klinefelter GR, Robert NL, Suarez JD. Direct effects of ethane dimethanesulphonate on epididymal function in adult rats. J Androl 1992;13:409-21.

Klinefelter GR, Suarez J, Roberts N, Strader L. The sperm biomarker SP22 is highly correlated with infertility resulting from the testicular toxicant bromochloroacetic acid. Biol Reprod 1999;187A(suppl).

Kohane AC, Cameo MS, Pineiro L, Garberi JC, Blaquier JA. Distribution and site of production of specific proteins in the rat epididymis. Biol Reprod 1980;23:181-7.

Kohane AC, Gonzalez EF, Pineiro L, Blaquier JA. Interaction of proteins of epididymal origin with spermatozoa. Biol Reprod 1980;23:737-42.

Lea IA, Richardson RT, Widgren EE, O'Rand MG. Cloning and sequencing of cDNAs encoding the human sperm protein, Spl7. Biochimica Biophysica Acta 1996;1307:263-6.

Linder RE, Klinefelter GR, Strader LF, Narotsky MG, Suarez JD, Roberts NL, Perreault SD. Dibromoacetic acid affects reproductive competence and sperm quality in the male rat. Fund Appld Toxicol 1995;28:9-17.

Linder RE, Klinefelter GR, Strader LF, Suarez JD, Roberts NL. Spermatoxicity of Dichloroacetic Acid. Reprod Toxicol 1997;11:681-8.

Linder RE, Klinefelter GR, Strader LF, Veeramachaneni DNR, Roberts NL, Suarez, JD. Histopathologic changes in the testes of rats exposed to dibromoacetic acid. Reprod Toxicol 1997;11:47-56.

Medical Research Council. IEH assessments on environmental oestrogens: consequences to human health and wildlife. Leicester, England: University of Leicester, 1995:105.

Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Ariga SMM, Ariga H. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem Biophs Res Comm 1997;231:509-13.

O'Hern PA, Bambra CS, Isahakia M, Goldberg E. Reversible contraception in female baboons immunized with a synthetic epitope of sperm specific lactate dehydrogenase. Biol Reprod 1995;52:331-9.

O'Rand MG, Irons GP, Porter JP. Monoclonal antibodies to rabbit sperm autoantigens. 1. Inhibition of in vitro fertilization and localization on the egg. Biol Reprod 1984;30:721-30.

O'Rand MG, Widgren EE. Identification of sperm antigen targets for immunocontraception: B-cell epitope analysis of Spl7. Reprod Fert Devel 1994;6:289-96.

O'Rand MG, Widgren EE, Richardson RT, Lea IA. Sperm antigen corresponding to a sperm zona binding protein autoantigenic epitope. US Patent #5,480,799, 1996.

Orgebin-Crist M-C, Danzo BJ, Davies J. Endocrine control of the development and maintenance of fertilizing ability in the epididymis. In: Hamilton DW, Greep RO, eds. Handbook of physiology, male reproductive system. Washington, DC: American Physiological Society, 1975:5(sect 7):319-38.

Peknicova J, Capkova J. Binding of boar spermatozoa to porcine oocytes: effect of low molecular weight 17-kDa protein. Mol Reprod Devel 1997;46:168-75.

Penttila TL, Yuan L, Mali P, Hoog C, Parvinen M. Haploid gene expression: temporal onset and storage patterns of 13 novel transcripts during rat and mouse spermiogenesis. Biol Reprod 1995;53:499-510.

Primakoff P, Lathrop W, Woolman L, Cowan A, Myles D. Fully effective contraception in male and female guinea pigs immunized with the sperm protein PH-20. Nature 1988;335:543-6.

Primakoff P, Woolman-Gamer L, Tung, KSK, Myles, DG. Reversible contraceptive effect of PH-20 immunization in male guinea pigs. Biol Reprod 1997;56:1142-6.

Ramarao CS, Myles DG, White JM, Primakoff P et al. Initial evaluation of fertilin as an immunocontraceptive antigen and molecular cloning of the cynomolgus monkey fertilin B subunit. Mol Reprod Devel 1996;43:70-5.

Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fert 1978;54:103-7.

Rochwerger L, Cuasnicu PS. Redistribution of a rat sperm epididymal glycoprotein after in vivo and in vitro capacitation. Mol Reprod Devel 1992;31:34-41.

Sharpe RM. Declining sperm counts in men - is there an endocrine cause? J Endocrinol 1993;136:357-60.

Shima S, Tziatzios C, Schubert D, Fukada H, Takahashi K, Ermler U, Thauer, RK. Lyotropic-salt-induced changes in monomer/dimer/tetramer association equilibrium of formyltransferase from the hyperthermophilic Methanopyrus kandleri in relation to the activity and thermostability of the enzyme. Eur J Biochem 1998;258:85-92.

Strader LF, Suarez JD, Roberts NL, Klinefelter GR. Spermatoxicity of bromochloroacetic acid (BCA) in the rat after 14 daily doses. The Toxicologist. 1998;51(lA).

Sullivan R, Boue F. Marker for male infertility and/or fertility. US Patent #5,723,305, 1998.

Toth GP, Zenick H, Smith MK. Effects of epichlorohydrin on male and female reproduction in long-Evans rats. Fund Appld Toxicol 1989;13:16-25.

Tsunoda Y, Chang MC. Fertilizing ability in vivo and in vitro of spermatozoa of rats and mice treated with a-chorohydrin. J Reprod Fert 1976;46:401-6.

Wagenfeld A, Ching-Hei Y, Strupat K, Cooper TG. Shedding of a rat epididymal sperm protein associated with infertility induced by Ornidazole and a-Chlorohydrin. Biol Reprod 1998;58:1257-65.

Wagenfield A, Gromoll J, Cooper TG. Molecular cloning and expression of rat contraception associated protein (CAP1), a protein putatively involved in fertilization. Bioch Biophys Res Cornm 1998;251:545-9.

Welch JE, Barbee RR, Roberts NL, Klinefelter GR. The sperm protein, SP22, is encoded by multiple transcripts in the rat testis. Biol Reprod 1999;339A(suppl).

Welch JE, Barbee RR, Roberts NL, Suarez JD, Klinefelter GR. SP22: a novel fertility protein from a highly conserved gene family. J Andrology 1998;19:393-5.

Wyrobek AJ, Gordon IA, Watchmaker G, Moore DHH. Indicators of genotoxic exposure. In: Bridges BA, Butterworth BE, Weinstein IB, eds. Banbury Report, Cold Spring Harbor, 1982:527-41.

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